Phospholipase D from Indian mustard seeds: Purification and enzymatic characterization

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Abbreviations

1,3 PC	1,3-Diacylglycerolphosphocholine
1,3-12:0/12:0 PC	1,3-Dilauroyl-*sn*-glycerol-3-phosphocholine
1,3-14:0/14:0 PC	1,3-Dimyristoyl-*sn*-glycerol-3-phosphocholine
1,3-8:0/8:0 PC	1,3-Dioctanoyl-*sn*-glycerol-3-phosphocholine
10:0 /10:0 PC	1,2-Didecanoyl-*sn*-glycero-3-phosphocholine
12:0/12:0 PC	1,2-Dilauroyl-*sn*-glycero-3-phosphocholine
14:0/14:0 PC	1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine
16:0/16:0 PC	1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine
16:0/18:1 PC	1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
18:0/18:0 PC	1,2-Distearoyl-*sn*-glycero-3-phosphocholine
18:1/18:1 PC	1,2-Dioleoyl-*sn*-glycero-3-phosphocholine
20:0/20:0 PC	1,2-Diarachidoyl-*sn*-glycero-3-phosphocholine
8:0/8:0 PC	1,2-Dioctanoyl-*sn*-glycero-3-phosphocholine
AlF_4^-	Aluminum fluoride
BRIJ-58	Polyethylene glycol hexadecyl ether
BSA	Bovine serum albumin
CMC	Critical micelle concentration
CTAB	Hexadecyltrimethylammonium bromide
EDTA	Ethylenediaminetetracaetic acid
HEPES	N-(2-Hydroxyethyl)piperazine-N´-(2-ethanesulphonic acid)
MES	2-(N-morpholino)ethanesulfonic acid
Na_3VO_4	Sodium orthovanadate
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol

PI	Phosphatidylinositol
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIPES	Piperazine-N,N′-bis(2-ethanesulfonic acid)
PLA1	Phospholipase A1
PLA2	Phospholipase A2
PLB	Phospholipase B
PLC	Phospholipase C
PLD	Phospholipase D
PMSF	Phenylmethylsulfonyl fluoride
P*p*NP	Phosphatidyl-*p*-nitrophenol
PS	Phosphatidylserine
SDS	Sodium dodecyl sulfate
TBS	Tris-Buffer-Saline
TBST	Tris-Buffer-Saline-Tween
TRIS	Tris(hydroxymethyl)aminomethane
TRITON X-100	4-Octylphenol polyethoxylate
TWEEN-20	Polyoxyethylene (20) sorbitan monolaurate
Lyso PC	Lysophosphatidylcholine

1. Introduction

Phospholipase D (PLD) (Phosphatidylcholin-phosphatidohydrolase, E.C. 3.1.4.4) is a ubiquitous enzyme that is widespread in both prokaryotic and eukaryotic systems. It has been implicated in a number of cellular processes such as transmembrane signaling, intracellular protein trafficking, secretion, alteration in cell morphology and motility (Morris *et al.*, 1997; Pappan *et al.*, 1997). The transphosphatidylation activity of PLD is utilized for the synthesis of rare natural phospholipids e.g phosphatidylserine (PS), phosphatidylglycerol (PG) and novel artificial phospholipids (Juneja *et al.*, 1987/1989; Takami *et al.*, 1994; Dippe *et al.*, 2008). Artificial phospholipids are interesting targets for both pharmaceutical and cosmetic applications. PLD obtained from plants or microorganisms has been used as a biocatalytic tool in the laboratory and on industrial scale for many years (Servi, 1999; Ulbrich-Hofmann, 2000; Ulbrich-Hofmann *et al.*, 2005).

At the beginning of research on PLD, the enzyme was used as a biocatalyst by chemists at laboratory scale for the synthesis of phospholipids by utilizing its transphosphatidylation potential (Dawson, 1967; Eibl and Kovatchev, 1981). At that time, PLD was used in the form of crude or partially purified extracts from plant sources such as from white cabbage (Dawson *et al.*, 1967; Yang *et al.*, 1967; Juneja *et al.*, 1987). It was only in 1990s when the multiple roles of PLD in plants (Wang, 2000; Bargmann and Munnik, 2006), yeast (Rudge and Engebrecht, 1999; Liscovitch *et al.*, 2000) and above all in mammalian cells (Exton, 2002; Jenkins and Frohmann, 2005) were recognized and the interest in PLD studies arose in biochemistry. Since then, there has been tremendous interest in the PLD study and today more than 1000 entries on PLD genes in GenBank (NCBI) and more than 6000 references on PLD in Scifinder Scholar (CAS) can be found. Thus it is not surprising that lately there has been a considerable and renewed interest in the enzymology of PLD.

Despite the importance and growing interest in the basic research and biocatalytic applications of PLDs, knowledge regarding their molecular properties, particularly their tertiary structure and conformational stability, is very limited. In spite of a great number of primary sequences of PLDs from mammals, microorganisms and plants have been elucidated on the DNA and mRNA level (Hammond *et al.*, 1995; Wang, 2000; Lerchner *et al.*, 2005), only a few of the enzymes could be obtained in pure state.

Plant PLD enzymes make up the largest group in the PLD superfamily (Selvy *et al.,* 2011). They have a highly significant contribution in the rich history of PLD superfamily in that the first description of a PLD enzyme was made in a plant source i.e. carrot (Hanahan and Chaikoff, 1947). Also, the first PLD enzyme was cloned from castor bean (Wang *et al.,* 1994). Furthermore, the PLD hydrolytic and transphosphatidylation activities were first demonstrated in plants in 1947 (Hanahan and Chaikoff, 1947) and in 1967 (Yang *et al.,* 1967; Dawson, 1967), respectively. In general, plant PLDs are monomeric and much larger than the microbial PLDs. Besides, the biochemical properties, domain structures and genomic organization of plant PLDs are much more diverse than those of other organisms (Qin and Wang 2002; Elias *et al.,* 2002; Wang, 2004). However, no crystal structure of plant PLD exist so far. The current model of proposed tertiary structure of the catalytic domain and reaction mechanism are based on the structure and characterization of two bacterial PLDs *Streptomyces* sp. Strain PMF (Leiros *et al.,* 2000; 2004) and *S. antiboticus* (Suzuki *et al.,* PDB code No. 2ZE4). PLDs from different sources show differences in their properties such as in their activity and stability and some of them possess uncommon substrate specificity in transphosphatidylation. As far as industrial applications are concerned only a few plant sources have been exploited for biocatalytical purposes. PLDs from selected Streptomyces strains are preferred due to the high yields of the transphosphatidylation product and the ready accessibility of the enzyme from bacterial culture media. Nevertheless, the use of plant PLDs become highly beneficial in the introduction of certain alcohols such as *myo*-inositol (Oblozinsky *et al.,* 2005). Thus, further progress in the enzyme-catalyzed phospholipid transformation will strongly depend on the availability of new PLD variants with high transphosphatidylation potentials.

In our previous study in India, PLD activity was identified in some new medicinally important plant sources i.e. *Brassica juncea* (mustard) seeds, *Zingibar officinale* (ginger) rhizomes, *Allium staivum* (garlic) bulbs and *Azadirachta indica* (neem) leaves. The aim of the study was to identify PLDs from unexplored plant sources, that possess high catalytic activity and stability (Khatoon *et al.,* 2007). It was found in the study that crude extract of mustard seeds possess the highest PLD specific activity as compared to other plant sources explored. Furthermore, unlike other plant PLDs, the PLD activity from crude extract of mustard seeds was found to possess high thermal stability i.e. it retained almost 55% activity even after exposure to 70°C for 30 mins. Also, the enzyme displayed maximum activity at millimolar levels of Ca^{2+} ions, however Ca^{2+} ions were not mandatory for the enzyme activity as the

enzyme also showed activity in the absence of Ca^{2+} ions. Moreover, the enzyme activity was not regulated by phosphatidylinositol-4,5-bisphosphate (PIP_2), an important regulator of PLD activity in both mammalian and plants system. Motivated by these findings, we became interested in characterizing mustard PLD in its purified form.

Thus, the present thesis represents the results of purification, molecular data and substrate specificity of PLD from mustard seeds. A comparison was made of the molecular and kinetic properties of mustard PLD to those of other plant PLDs available in literature. As PLD, like other lipolytic enzymes is interfacially activated, the kinetic characterization of mustard PLD was performed in two different assay systems, in a mixed-micellar assay and in an aqueous-organic two-phase assay system. Because PLD from plant sources display a strong activation by metal ions, the effect of metal ions in general and Ca^{2+} ions in particular, on the activity and stability of mustard PLD was studied. In addition, the influence of pH and temperature on the activity and stability of the enzyme was performed. Furthermore, the effect of activators like PIP_2, organic solvents, detergents and inhibitors like aluminum fluoride (AlF_4^-) and sodium orthovanadate (Na_3VO_4) was investigated. Finally, the transphosphatidylation potential of the enzyme in comparison to its hydrolytic activity was evaluated.

2. Theoretical part

2.1. Types of phospholipases and their substrates

Phospholipids are present in all living organisms. They are major constituents of all biological membranes in addition to glycolipids and cholesterol. Enzymes targeted at modifying these phospholipids by hydrolysis are called phospholipases. Phospholipases constitute a complex and crucially important class of lipolytic enzymes that catalyze the hydrolysis of the phospholipids at their ester or phosphodiester bonds. These enzymes play an important 'housekeeping' role in phospholipid catabolism and turnover associated with membrane synthesis, degradation, and organelle biogenesis and can also generate molecules with important biological activities by degradation of otherwise inert phospholipid substrates (Morris *et al.*, 1997). In biocatalysis also, phospholipases have been exploited for a number of phospholipid transformation reactions in the laboratory as well as industrial scale (Servi, 1999; Ulbrich-Hofmann, 2000; De Maria *et al.*, 2007).

According to their site of hydrolysis on glycerol-phospholipids, phospholipases can be classified into phospholipase D, C, A_2, A_1 and B (fig 2.1). Phospholipase A1 and A2 (PLA1 and PLA2) catalyses the hydrolysis of ester bond in *sn*-1 and *sn*-2 position respectively. Phospholipase C (PLC) catalyzes the hydrolysis of phsosphodiester bond at the glycerol side and phospholipase D (PLD) at the polar side. On the other hand, phospholipase B (PLB) hydrolyzes the glycerophospholipids in both the *sn*-1and *sn*-2 position (Saito *et al.*, 1991; Oishi *et al.*, 1996/1999; Merckel *et al.*, 1999).

Some special types of these enzymes possess additional specificity with regard to the alcoholic head group of phospholipids such as the glycophosphatidylinositol (GPI)-specific PLD or the phosphatidylinositol (PI) specific PLC (PI-PLC). The former hydrolyzes the GPI anchors to which some proteins are anchored and inserted into the membrane. Other related enzymes are lysophospholipases which specifically hydrolyze either 1-lysophospholipids or 2-lysophospholipids (Karasawa and Nojima, 1991; Waite, 1987). In addition to this there is sphingomylinase, regarded as a phospholipase similar to PLC which hydrolyzes sphingomyelin to N-acylsphingosine and choline phosphate (Ikezawa *et al.*, 1978; Levade and Jafferezou, 1999).

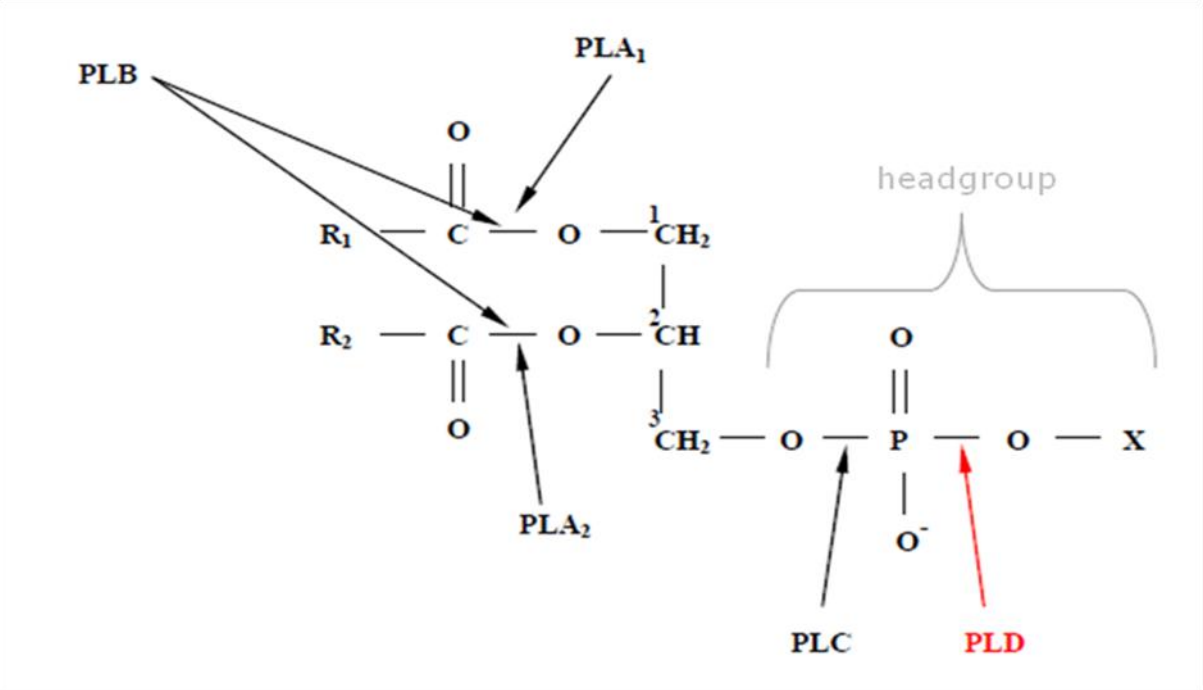

Fig. 2.1. Hydrolytic sites of Phospholipases A1, A2, B, C and D on a phospholipid molecule. The site of hydrolysis for each phospholipase is indicated with an arrow.

Phospholipids as substrate of phospholipases

Phosphoglycerides or glycerophospholipids are derivatives of glycerol in which at least one of the two hydroxyl groups of the glycerol backbone (C1 and / or C2) are esterified to the carboxyl groups of the fatty acid chains (saturated or unsaturated) and the third hydroxyl group is esterified to phosphoric acid. The simplest phosphoglyceride is diacylglycerol-3-phosphate or phosphatidate. Other phosphoglycerides are derived from phosphatidate by esterification of its phosphate group to the hydroxyl group of several alcohols such as choline, ethanolamine, inositol, serine or glycerol. Depending on the structure of the alcoholic head group, the glycerophospholipids can be charged or uncharged, thus at neutral pH of 7.0, the glycerophospholipids composed of positively charged alcoholic group (choline and ethanolamine) are zwitterionic and neutral (phosphatidylethnolamine (PE) and phosphatidylcholine (PC)), and the one having uncharged (glycerol and inositol) or zwitterionic (serine) alcoholic head group behave as negatively charged and acidic molecules (phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidic acid (PA) and phosphatidylserine (PS)).

Phospholipids are amphiphilic in nature i.e. they possess both hydrophilic or polar regions and hydrophobic or non-polar regions on the same molecule. Natural phospholipids are insoluble

in water. On interaction with water, the polar groups due to their solvation, try to keep the hydrocarbon residues in the aqueous medium so as to prevent the phase separation of the phospholipid and water. Thus, in order to minimize the unfavorable water-hydrocarbon interface, the phospholipid molecules arrange themselves at the air-water interface to form a **monomolecular layer** (fig 2.2f), with the polar head groups residing in the aqueous phase and the non polar tail hanging in the air. Alternatively, the phospholipids can spontaneously self-assemble to form higher molecular aggregates in which the hydrophilic moieties are solvated at the surface of the aggregate resulting in the sequestration of hydrophobic moieties from water. The type of aggregates formed depends not only on the chemical structure of the phospholipid but also on physical parameters such as surface charge, temperature, the medium including ions and surfactants (Ulbrich-Hofmann, 2000).

Fig 2.2 shows the various polymorphic phases formed by phospholipids in aqueous environment. The parameter that influences the type of aggregate formed is the ratio between the polar and non polar moieties in the phospholipid molecule. For instance when cylindrically shaped molecules with the predominant hydrophobic moiety (such as PC) (fig 2.2I), is brought in contact with water, it forms **multilamellar vesicles (MLV)** also called liposomes. Liposomes are composed of topologically closed and concentrically arranged phospholipid bilayers separated from each other by layers of aqueous medium. They are generally 0.1 to 5 µm in size (fig 2.2a). Upon treatment with ultrasonic radiation, MLVs can be transformed into small unilamellar vesicles (SUVs) (Fig 2.2b), with a highly curved outer surface and a diameter of not less than 20-30 nm. In another case, the cone-shaped molecules with the predominant hydrophilic moiety (fig 2.2II) resulted due to the shortening of fatty acyl chain length or reduction in the number of fatty acyl chains in the molecule (e.g. LysoPC) are generally soluble in water at low concentrations. But if their concentration surpasses a critical concentration, called as critical micellar concentration (cmc) which is in the range of 10^{-10} M, molecules began to aggregate into spherical or rod shaped resulting in the formation of so called **micelles** (fig 2.2d) (Walde *et al.*, 1990; Chopineau *et al.*, 1998). Micellar solutions are optically clear and the lipid molecules in the micelle are in rapid equilibrium with the molecules in free solution. The micelles differ from vesicles in that the large polar parts of the molecule are oriented towards the water phase and the non polar fatty acid chains congregate to form a non polar interior. Furthermore, when phospholipids interact with organic solvents, they can produce **reverse micelles** (fig 2.2d) with their hydrophilic head groups assemble to form a polar interior. The interior of reverse micelles is able to carry water or hydrophilic

molecules like salts or even proteins. Finally, the phospholipid molecules having the shape of an inverted truncated cone (fig 2.3III) with the predominant hydrophobic moiety (such as PE) preferably form **hexagonal structures** (fig 2.2c). The hexagonal structures differ from vesicles in that the aqueous phase is surrounded by lipid monolayer, while in vesicles the aqueous interior is bordered by a bilayer (Eibl, 1984).

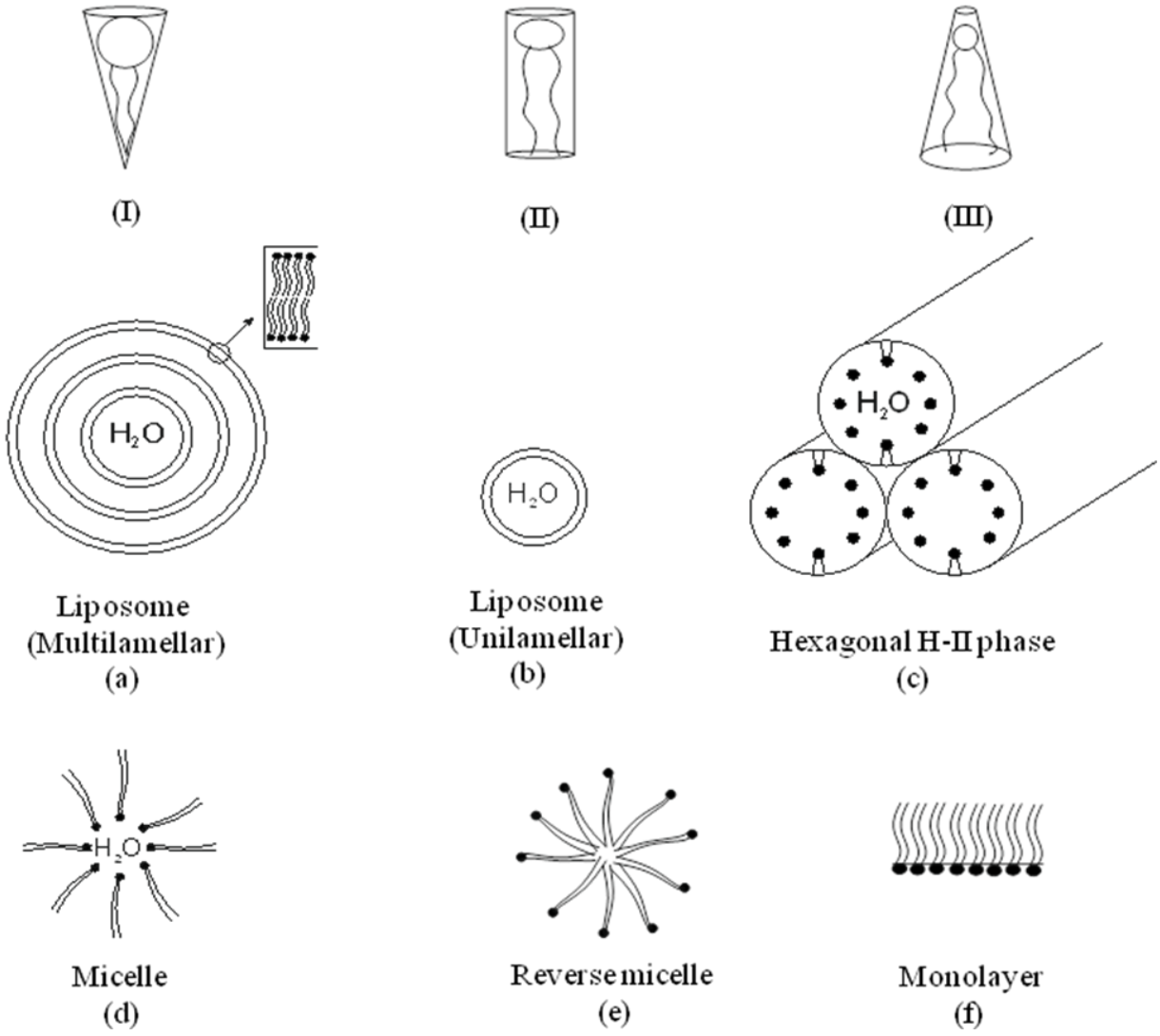

Fig 2.2 The various polymorphic phases formed by phospholipids in aqueous environment. Figures I-III demonstrate the geometry of phospholipids: (I) cone; (II) cylinder; (III) inverted cone. Figures a-f demonstrate the supramolecular structures of phospholipid molecules (adapted from Ulbrich-Hofmann, 2000).

For a particular phospholipid with a given headgroup, the variation in physical, chemical, and biological properties is the result of the wide variation of fatty acyl chain compositions. Along with chain length, the degree of unsaturation of the lipid tails also has great influence on the properties of phospholipid molecule. The introduction of double bond produces a kink in the

alkane chain, resulting in a greater mobility of the molecule and disrupting the regular periodic structure. It is this disruption that leads to decrease in phase transition temperatures (T_m) from the crystalline (ordered) to liquid crystalline (fluid) state of the phospholipids molecule. While the hydrocarbon chains are tightly packed in the gel state, they are loosened up in the fluid phase, and this loosening is associated with the lateral expansion of lipid bilayers.

Table 2.1: Selected physical properties of diacylglycerophosphocholine Aggregation state of phospholipids actually depends upon their concentration in water. T_m is thermotropic phase transition (Lichtenberg *et al.,* 1983).

Acyl chain length	PC name	State in water	T_m (°C)
2	Diacetyl PC	Monomer	-
3	Dipropionyl PC	Monomer	-
4	Dibutyroyl PC	Monomer	-
6	Dihexanoyl PC	Micelle	-
7	Diheptanoyl PC	Micelle	-
8	Dioctanoyl PC	Micelle	-
12	Dilauroyl PC	Bilayer	0
14	Dimyristoyl PC	Bilayer	23
16	Dipalmitoyl PC	Bilayer	41
18	Distearoyl PC	Bilayer	58
22	Dibehenoyl PC	Bilayer	75
18:1	Dioleoyl PC	Bilayer	-22
Mixture	Egg PC	Bilayer	-11

As discussed above, the substrate of phospholipases i.e. phospholipids form complex structure in water, therefore making the investigation of the kinetic behaviour of phospholipases is much more difficult than that of the hydrolytic enzymes whose substrates are water soluble.

One of the most important characteristic and intriguing features of lipolytic enzymes is their "activation" by interfaces i.e these enzymes are more active towards their substrates in aggregated form than in monomolecular forms. Because of interfacial activation, the phospholipases activities not only depend on the substrate and enzyme concentrations but also on the physical state of substrate i.e. on the organization and dynamics of the interface where catalysis occurs. Thus, one of the most important prerequisite for kinetic measurements is the preparation of reproducible substrate aggregates. The natural substrates of phospholipases are

long-chain phospholipids that preferentially form aqueous dispersions (fig 2.2a,b). Therefore, the kinetics of the enzymes cannot easily be studied in such a system. For the type of phospholipid substrate shown in fig 2.2a, it is difficult to kinetically analyze their hydrolysis as enzyme is accessible to unknown number of substrate molecules and therefore even qualitative conclusions are limited.. Therefore, quantitative and reproducible kinetic measurements of phospholipase hydrolysis on natural long-chain phospholipids can only be made with micellar system, where each phospholipid molecule is present at the interface and in principle, is accessible for the enzyme attack. The two ways which so far have proved to be particularly useful to achieve the transformation of the liposomes into micellar systems are: (i) the use of organic solvents or (ii) detergents. (Verger and Haas, 1976).

The first technique involves the use of a water-immiscible solvent such as diethylether, which when added to the lipid-bilayer in water, resulted in the formation of micro-dispersions or inverted micelles where the contact between the substrate and enzyme takes place at the interface. In biocatalytic applications, the reactions are mostly carried out in shaken emulsion systems, where a non polar organic solvent such as diethyl ether containing the dissolved phospholipid is mixed with an aqueous phase containing the buffer and the enzyme (Ulbrich-Hofmann, 2000). **The second technique** is based on the addition of detergents in an appropriate amount which resulted in transformation of the lipid bi-layer structure into an optically clear solution of phospholipid-detergent mixed micelles. The state of aggregation in a mixture of phospholipids and detergent depends on the ratio of the two components as well as on the temperature, ionic strength etc. Such phospholipid-detergent mixed-micelles are readily attacked by phospholipases and result in well reproducible kinetic measurements. However, complexity of the additional component (i.e detergent) and its possible influence on the enzyme structure must be taken into account.

The **kinetics of interfacial activation** has been explained using various approaches. Verger et al. (1973) formulated the principle of enzyme activation by interfaces, whereby they proposed that the binding of the enzyme to the interface transforms it into its activated form which in turn binds to the substrate molecule and forms the product in a normal catalytic way. This model has been characterized for the PLA2 reaction on monolayer. Another model called surface dilution model (Deems *et al.,* 1975; Dennis, 1994) is based on the assumption that the water soluble enzyme first binds to one phospholipid molecule present in an interface to form enzyme-substrate complex followed by binding of second substrate molecule in its catalytic

site to form the Michaelis enzyme-substrate-substrate complex which finally formed product via the enzyme-substrate-product complex. This model has been derived from the kinetic data of the action of cobra venom PLA2 in mixed micelles with the nonionic surfactant Triton X-100. Both models yield mathematically equivalent kinetics and are appropriate to fit the experimental results.

2.2. Phospholipase D

PLD catalyzes the hydrolysis of phosphoglycerides at the terminal phosphodiesteric bond, producing phosphatidic acid (PA) and the alcohol released from the hydrophilic head group. In addition to hydrolysis, it can also transfer the phosphatidyl moiety to appropriate acceptor alcohols to form phosphatidylalcohols, through a process referred to as transphosphatidylation (fig 2.3).

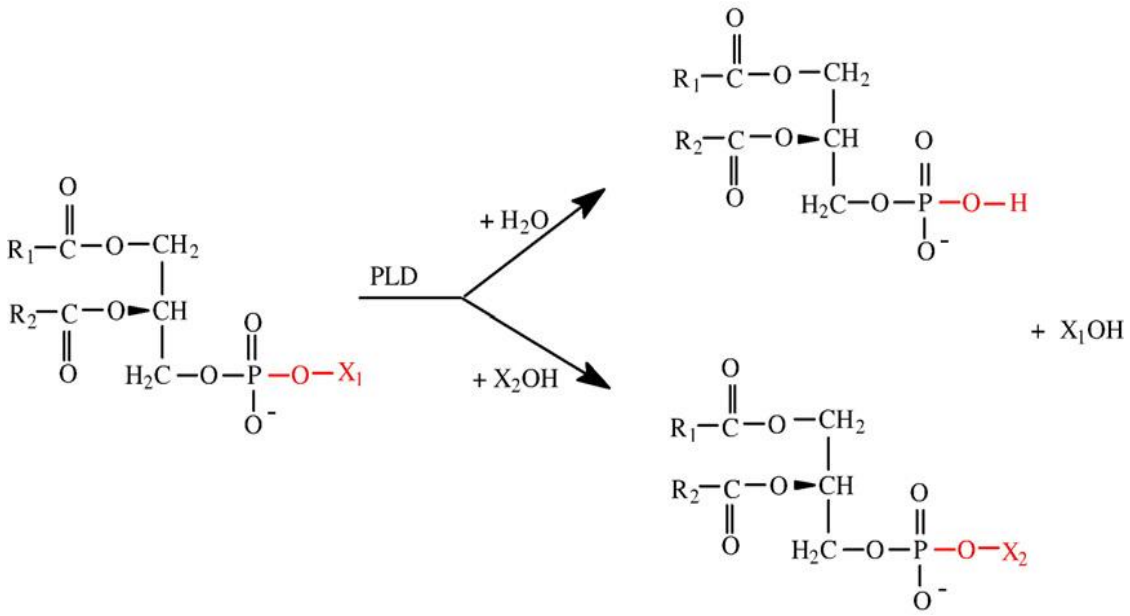

Fig. 2.3. Reaction scheme for the PLD-catalyzed hydrolysis and transphosphatidylation of a 1,2-diacyl-*sn*-glycero-3-phosphocholine. R_1 and R_2 denote the alkyl residues of the acyl residues in *sn*-1 and *sn*-2 position. X_1OH and X_2OH represent the released alcohol and the acceptor alcohol.

From literature studies it is now evident that PLD is a ubiquitous enzyme and its activity is present in all types of organisms ranging from viral system (Poxviridae family), microbial system (Arcano-, Corynbacterium, *Vibrio damsela*, many *Streptomyces*-Species, *Saccharomyces cerevisiae*, *Streptoverticillium cinnamoneum* etc) animal system (for e.g. Drosophila, Nematodes, Rat, Mouse, Hamsters, Cattle, Humans) to plant system (for e.g. *Arabidopsis thaliana*, carrots, different types of cabbage, tomato, peanuts, rice, maize, tobacco, cotton, soyabeans, castor beans, sunflower, poppy etc) (Vaskovsky *et al.*, 1972; Heller, 1978; Dennis, 1983; Waite 1987; Ponting and Kerr, 1996; Morris *et al.*, 1997;

Abousalham *et al.,* 1999; Oblozinsky *et al.,* 2003). In some organisms several isoenzymes of PLD have been discovered, which are found in various stages of development (Oblozinsky *et al.,* 2003) or are limited to certain tissue types (Dyer *et al.,* 1994/1996). They differ in their properties with respect to molecular mass, pH optimum or Ca^{2+} dependence and its regulation.

2.2.1. Structure and stability of PLDs

2.2.1.1. Molecular organization and relationship among PLDs

Based on the determination of the amino acid sequences of the enzymes possessing PLD activity, a large family of homologous proteins was defined known as PLD superfamily (Mansfeld and Ulbrich-Hofmann, 2009). The PLD superfamily, in addition to PLDs, also includes other proteins like cardiolipin synthases and phosphatidylserine synthases from E.*coli* (Morris *et al.,* 1996), endonuclease Nuc from Salmonella typhimurium, poxvirus envelope proteins, murine toxin from *Yersinia pestis* (Ponting and Kerr, 1996) and human tyrosyl-DNA phosphodiesterase Tdp1 (Interthal *et al.,* 2001). The most common feature of the members of the PLD superfamily is the presence of two highly conserved, similar but not identical HxKxxxxDxxxxxxG(G/S) motifs. These so-called HKD motifs are essential for catalysis (Sung *et al.,* 1997; Lerchner *et al.,* 2006). They form one catalytic site as revealed from the crystal structure of PLD from *Streptomyces* sp. strain PMF (Leiros *et al.,* 2000; Stukey and Dixon, 1999; Davies *et al.,* 2002). The HKD motifs belong to a total of four homologous regions (I-IV) that could be identified in PLD genes from plants, yeast and animals. Bacterial genes possess only two conserved homologous regions (II and IV) corresponding to two HKD motifs (fig 2.4). The function of the other two homologous regions (I and III) is not yet understood. However, mutational studies on a human PLD suggest that they are also involved in catalysis (Sung *et al.,* 1997). In comparison to bacterial PLDs, which are generally extracellular and bear N-terminal signal sequences (Iwasaki *et al.,* 1994), the PLDs from plants, yeast and mammals are characterized by further homologous sections in the N-terminal region. So, PLDs from mammals and yeast as well as ζ-type from Arabidopsis are characterized by Phox (PX) and Pleckstrin (PH) domains in the N-terminal region (Frohman and Morris, 1999; Qin and Wang, 2002), whereas most plant PLDs carry Ca^{2+}- dependent phospholipid binding domain also called as C2 domain in their N-terminal region. A common feature of both mammalian and plant PLDs is an absolute requirement of intact C-terminus (Xie *et al.,* 2000 and Liu *et al.,* 2001; Lerchner *et al.,* 2006).

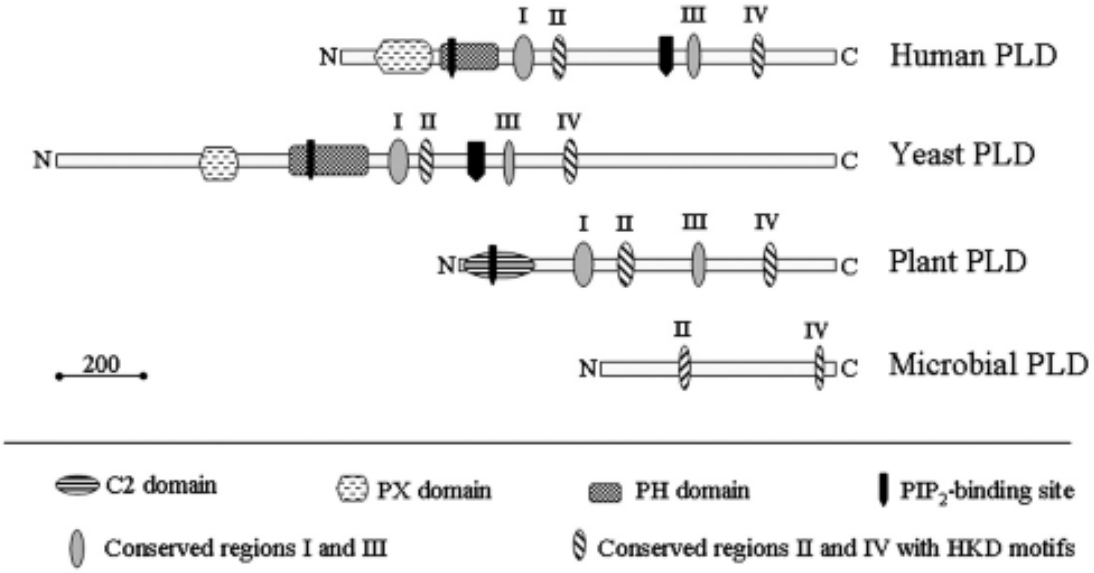

Fig. 2.4. Schematic presentation of domain structures of PLD as representatives from different sources. CLUSTAL Win the programme MEGA 4 is used to perform the alignment of the protein sequences. The linear extension of the symbols corresponds to the length of the corresponding motif. The representative shown in the figure for plant PLD is PLDα1 from *Arabidopsis thaliana*, for human is PLD1 from homo sapiens, for yeast is PLD1 from *Saccharomyces cerevisiae* and for microbial PLD is the PLD from *Streptomyces antibioticus* (taken from Mansfeld and Ulbrich-Hofmann, 2009).

Interestingly, the common α-type plant PLDs as well as the *Streptomyces* PLDs possess highly conserved cysteine residues. In extracellular microbial PLDs, these conserved cysteine residues form disulphide bridges located in regions at the surface (Leiros *et al.*, 2000), whereas, the 8-9 cysteine residues in the plant enzymes of α-type are fully reduced (Hwang *et al.*, 2001). It was concluded from the mutational studies on cabbage PLD2 that all the highly conserved cysteine residues, particularly Cys310 and Cys625 near the two HKD motifs, are involved in the preservation of local structural elements and/or in the binding of acceptor molecules (Lerchner *et al.*, 2006). The role of cysteine residues in mammalian PLDs for e.g. 12 and 14 in human PLD1 and PLD2 and 13 and 14 in rat PLD1 and PLD2 is not yet clear though they are also well conserved. In comparison to plant and mammalian PLDs, the PLDs from yeast and filamentous fungi are not yet very well studied. The enzyme cloned from *Aspergillus nidulans* contains all the four conserved regions I-IV but possess neither the C2 domain as in most plant PLDs nor PH and PX domains which are the characteristic of mammalian and yeast PLDs (Hong *et al.*, 2003).

2.2.1.2. Isoforms and classification of PLDs

As compared to other eukaryotic system, the family of plant PLDs is much more diverse as far as the genomic organization, domain structure and biochemical properties are concerned

(Qin *et al.*, 2002; Elias *et al.*, 2002 and Wang, 2004). Thus, 12 PLD isoforms are identified in *Arabidopsis thaliana* (Qin and Wang, 2002), while 17 PLD isoforms are derived from the genomic DNA in rice (Li and Xue, 2007). Several PLD isoenzymes can also be found in GenBank (NCBI) for other plants e.g. cabbage, poppy, castor bean, tomato, cotton, and mosses. In case of mammals, two types of well known PLDs (PLD1 and PLD2) that share about 50% identity and differ in basal and regulatory interactions are recognized. A novel interesting PLD occurring in mitochondria has also been described (Choi *et al.*, 2006). This enzyme which occurs in dimeric form contains one HKD motif per monomer and is very similar to the bacterial endonuclease Nuc and cardiolipin synthase. In most *Streptomyces* strains only one secreted PLD occurs.

As stated above, the plant PLD family is comprised of multiple PLDs with distinctively different biochemical, molecular and structural properties (Li *et al.*, 2009). Thus, the 12 PLD genes in Arabidopsis have been classified into six types based on gene architecture, sequence similarity, domain structure, biochemical properties and the order of cDNA cloning as: PLDα (3), β (2), γ (3), δ, ε and ζ (2) (Qin and Wang, 2002). Based on the overall protein domain structure, these PLDs are further divided into two subfamilies, the C2-PLDs and PH/PX-PLDs. Ten of the 12 PLD isoenzymes (α-, β-,γ-, δ- and ε types) contain one C2 domain in their N-terminal region and are characterized as Ca^{2+}-dependent or C2-PLDs whereas the two PLDs of ζ-type (PLDζ1 and PLDζ2) contain N-terminal PX and PH domains like the mammalian PLDs and are characterized as Ca^{2+} independent or PH/PX-PLDs. Further, based on the requirements for Ca^{2+} and lipid activators in *in vitro* assays, PLDs can be grouped into three classes: (a) the conventional PLD that is most active at millimolar levels of Ca^{2+} (20 to 100 mM), (b) the polyphosphoinositide dependent PLD that is most active at micromolar levels of Ca^{2+}, and (c) the phosphatidylinositol (PI) specific PLD that is Ca^{2+}-independent (Wang, 2000). The α-type is the most prevalent and best studied class in plants. Members of this class have been purified to apparent homogeneity from several plant sources (Wang, 2000). Multiple PLDαs have been cloned from cabbage (Kim U *et al.*, 1999; Pannenberg *et al.*, 1998; Schäffner *et al.*, 2002), poppy (Lerchner *et al.*, 2005), castor bean *Craterostiga plantagineum* (Frank *et al.*, 2000), and rice (Morioka *et al.*, 1997). Moreover, PLDβ and PLDγ are PIP_2dependent and most active at micromolar levels of Ca^{2+}, while the PI-specific PLD was identified in suspension cells of *Catharanthus roseus* (Wissing *et al.*, 1996).

2.2.1.3. Tertiary structure and catalytic mechanism

So far little information in the literature is available on the tertiary structure of eukaryotic PLD. The lack of progress in this area might be attributed to the strong propensity of larger eukaryotic PLDs to aggregate in concentrated solutions as observed from the attempts to crystallize cabbage PLD (Ulbrich-Hofmann *et al.,* 2005). Moreover, due to the large differences in size and low degree of identity at molecular level, it is difficult to interpret the structure of eukaryotic PLD from the information available on the crystal structure of only two bacterial PLDs i.e. PLD from *Streptomyces* strain PMF (Leiros *et al.,* 2000; 2004) and PLD from *S. antibioticus* (Suzuki *et al.,* PDB code No. 2ZE4). Nonetheless, there is consensus that the active sites of all PLDs belonging to the PLD superfamily are probably similar (Mansfeld and Ulbrich-Hofmann, 2009). The structure of the two bacterial PLDs described above is shown in fig 2.5. They are monomeric proteins consisting of two domains of similar topology with an α-β-α-β-α-sandwich structure. The eighteen α-helices flanked the two β-sheets that are formed by nine and eight β-strands. Moreover, the structure consists of several extended loop regions. One of these loops comprising the residue 382-389 (according to the amino acid sequence of *Streptomyces* sp. strain PMF) is conserved in PLDs of most *Streptomyces* species and is suggested to be the interfacial binding region (Leiros *et al.,* 2004). At the interface of the two structural domains, the active site region with the highly conserved HKD motifs is localized. Recently, the initial studies carried out on the shape and conformation of cabbage PLD2 by small-angle X-ray scattering and CD spectroscopy suggested the structure for α-type PLD as a barrel-shaped monomer with loosely structured tops and dominant β-structural elements (Stumpe *et al.,* 2007). Besides, a model of the catalytic core has also been derived for the mammalian PLD1 (Exton, 2004).

The catalytic mechanism for PLD is suggested to be of ping-pong type with a covalent phosphatidyl-enzyme intermediate (Ulbrich-Hofmann, 2000). This mechanism is further confirmed by Leiros et al. (2004) from the analyses of crystal structures of PLD from *Streptomyces* sp. strain PMF in the presence of both short chain substrate and product.

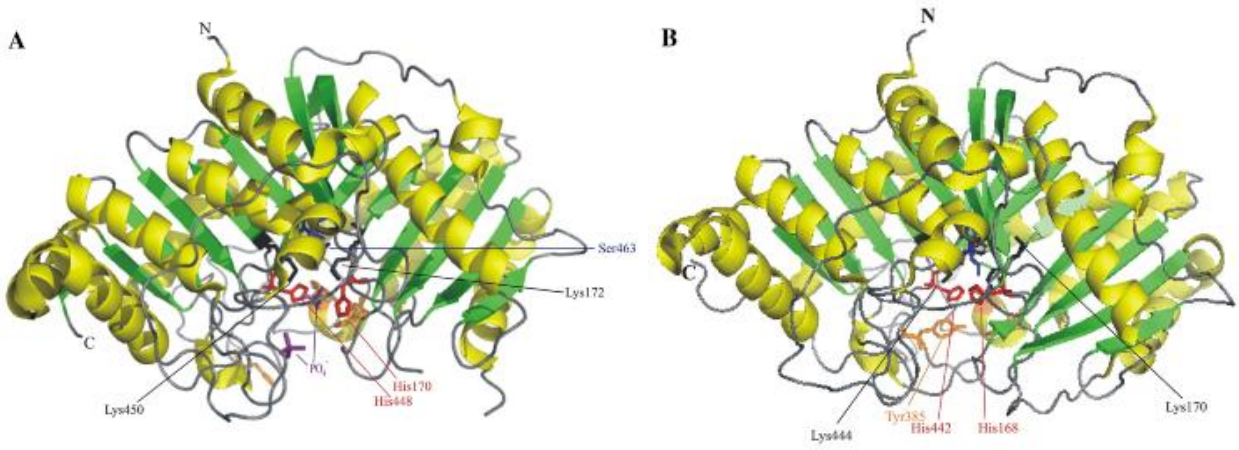

Fig. 2.5. **Tertiary structures of (A) PLD from Streptomyces sp. strain PMF (PDB code No. 1F0I) and (B) from PLD from *Streptomyces antibioticus* (PDB code No. 2ZE4) edited with PyMol.** Helices are shown in yellow, ß-sheets in green, loops in grey. Red symbolizes the His residues of the two HKD motifs, black the Lys residues of the two HKD motifs. Blue is used to highlight the GG and GS sequence motifs of the two HKD motifs, deep purple indicates the two phosphate residues encountered in the crystal structure. Amino acids shown in orange were described to be involved in substrate recognition/binding and selectivity in PLDs from *Streptomyces seseptatus* and *Streptomyces antibioticus* (adapted from Mansfeld and Ulbrich-Hofmann, 2009).

2.2.1.4. Stability of PLDs

As for now, only limited knowledge on the stability of PLD is available from the literature. It was described from the thermostability studies on PLDs from several *Streptomyces* species that one amino acid present in the hinge region is critical for the stability of the whole molecule (Hatanaka *et al.*, 2002; 2004 and Negishi *et al.*, 2005). For PLDα2 from cabbage, flexible regions were determined by limited proteolysis within the C2 domain and also close to the N-terminal side of the first HKD motif (Younus *et al.*, 2003). Moreover, conformational studies on cabbage PLDα2 showed that it has a strong aggregation propensity and that unfolding of protein proceeds through a globule like intermediate (Sussane Haufe, 2006). Interestingly, Ca^{2+} ions though essential for activity were shown to destabilize PLDα2 from cabbage (Stumpe *et al.*, 2007).

2.2.2. Production and catalytic properties of plant PLDs

2.2.2.1. Purification of PLD from plants

PLD exists in soluble as well as in membrane bound form. Several methods were employed to purify the soluble enzymes from plant sources (Davidson and Long, 1958; Yang *et al.*, 1967; Allgyer and Wells, 1979; Heller *et al.*, 1974; Wang *et al.*, 1993). The biochemical and structural characterization of plant PLD enzymes has been hindered by their instability during and after purification from plant tissues (Allgyer and Wells, 1979; Abousalham *et al.*, 1999)

or the low yields in recombinant protein production (Wang *et al.*, 1994), respectively. In a study with the immobilization of PLD, it was found that Ca^{2+} play a specific role in the adsorption of PLD *via* hydrophobic interaction to the polymeric carriers containing long chain anchor groups such as octadecyl, octyl, or other alkyl residues (Lambrecht and Ulbrich-Hofmann, 1993). Simultaneously, these results were used to develop a more efficient procedure of PLD purification based on Ca^{2+} mediated specific binding of PLD onto the hydrophobic support of Octyl-Sepharose CL-4B (Lambrecht and Ulbrich-Hofmann, 1992). Since then this procedure is widely and successfully used as one of the crucial step for the purification of PLDs from different plant sources such as cabbage (Abousalham *et al.*, 1993), soybean (Abousalham *et al.*, 1995), rice (Morioka *et al.*, 1997), rape (Novotna *et al.*, 1999), poppy (Oblozinsky *et al.*, 2003) and peanut (Nakazawa *et al.*, 2006). It also proved highly useful for the one-step purification of recombinantly expressed PLDs such as cowpea PLD (El Maarouf *et al.*, 2000), PLDα1 and PLDα2 from cabbage (Schaeffner *et al.*, 2002), PLD1 and PLD2 from poppy (Lerchner *et al.*, 2005).

Molecular masses of plant PLDs from different sources, as determined by gel filtration, sedimentation or SDS-PAGE, strongly vary and depend on whether they were determined under native or denaturing conditions. They range from 78-200 kDa (Heller, 1974/1978; Allgyer and Wells, 1979; Lambrecht and Ulbrich-Hofmann, 1992; Abousalham *et al.*, 1993; Wang *et al.*, 1993; Oblozinsky *et al.*, 2003). As far as the amino acid sequences are known, more exact molecular masses can be calculated. They are in the range of 90-95 kDa for most plant PLDs (Schoeps *et al.*, 2002; Lerchner *et al.*, 2005.). In general, PLDs are monomeric but there also reports on polymerization (Abousalham *et al.*, 1999). The isoelectric point (pI) of PLDs ranges from 4.2-8.3 (Abousalham *et al.*, 1999; Qin *et al.*, 2006).

2.2.2.2. Activation by metal ions

Most plant PLDs require Ca^{2+} ions in micromolar or even millimolar concentrations ranges for activity, when analyzed in vitro. This requirement for Ca^{2+} ions is related to the C2 domain at the N-terminal region of most plant PLDs. The C2 domain comprises approximately 130 residues forming an eight stranded antiparallel *β*-sandwich (Nalefski and Falke, 1996). In comparison to α-type PLD that require non-physiologically high concentrations of Ca^{2+} ions (20-200 mM), the PIP_2dependent PLDβ and PLDγ require micromolar concentrations of Ca^{2+} ions (Wang, 2000). Moreover, PLDβ and PLDγ catalysis is inhibited by high millimolar Ca^{2+} ions concentration (Wang, 2000). For the separately

expressed His-tagged C2 domains of PLDα and β from *A. thaliana*, 1-3 low affinity binding sites (K_D values in the range of 0.59-0.47) and three high affinity binding sites (one with K_{D1} = 0.8 and two with K_{D2} = 24 μM) for Ca^{2+} ions were derived (Zheng *et al.,* 2000). However, it has been shown for PLDβ from *A. thaliana* that Ca^{2+} ions can also interact with the catalytic regions of the enzyme via several binding sites which act cooperatively on activity (Pappan *et al.,* 2004). From the near UV CD signals and activity data, two binding events with the differences of two orders of magnitude (K_D values of 0.1 mM and 10-20 mM) were evaluated for α–type PLD2 from cabbage (Stumpe *et al.,* 2007). It was proposed that the higher requirement of Ca^{2+} ions by PLDα in comparison to PLDβ is caused due to the substitution of two of the well-conserved acidic Ca^{2+}-coordinating amino acid residues by positively charged or neutral amino acids (Pappan *et al.,* 1997). Also, the requirement of millimolar level of Ca^{2+} ions by PLDα, is independent of the substrate and its aggregation state (Kuppe *et al.,* 2008) and this finding eventually rule out the argument that the activation of α-type PLD by such high concentrations of Ca^{2+} ions is merely a medium effect. Interestingly, an α–type PLD which was 4-fold activated by Zn^{2+} ions than by Ca^{2+} ions was isolated from poppy seedlings (Oblozinsky *et al.,* 2003).

2.2.2.3 Influence of pH

PLD from plants are reported to display a wide range of pH optimum (Heller, 1978). In most cases it is observed that the pH optimum of plant PLDs falls in the weak acidic range for instance pH 5.7 for cabbage PLD (Lambrecht and Ulbrich-Hofmann, 1992), pH 5.5-6.0 for rape seed (Novotna *et al.,* 1999), pH 5.0 for peanut PLD (Nakazawa *et al.,* 2006). Interestingly, an uncommon PLD from poppy seedlings which is activated by Zn^{2+} ions displayed a pH optima of 8.0 (Oblozinsky *et al.,* 2003). Besides, it is observed that Ca^{2+} ions have a strong influence on the pH optima of α-type plant PLDs. Thus, soybean PLD demonstrated a shift in optimal activity at pH 7.5 with 10 mM Ca^{2+} to pH 5.5 with 40 mM Ca^{2+} (Abousalham *et al.,* 1995). Similarly, shift in pH optima with Ca^{2+} ions concentration was also observed with PLDα from castor bean (Pappan and Wang, 1999), PLDα from cabbage (Abousalham *et al.,* 1993) and recombinantly expressed PLDα1 and PLDα2 from poppy (Lerchner *et al.,* 2005). However, with recombinantly expressed cabbage PLDα1 and α2, no dependence of Ca^{2+} ions on the pH optima was observed (Schaeffner *et al.,* 2002).

2.2.2.4 Activation by detergents and organic solvents

Anionic amphiphiles are strong activators of plant PLDs (Heller, 1978; Dawson and Hemmington, 1967). Sodium dodecyl sulphate (SDS) is mostly employed as detergent to prepare substrate vesicles. For instance, with cabbage PLD, the activation effect of SDS with long chain PC substrate is 18 fold as compared to 3.5 fold with the micellar short-chain substrate 1,2-dihexanoyl-*sn*-glycerophosphocholine (Dittrich *et al.,* 1998). Other anionic surfactants such as 1,3-didodecanoylglycero-2-sulphate or 1,3-didodecanoylglycero-2-phosphate also displayed activation effects on cabbage PLD (Dittrich *et al.,* 1998). These activation effects are probably not specific but related to the charge and structuring of the substrate aggregates (Mansfeld and Ulbrich-Hofmann, 2009). Also, from the studies on PLD in Triton X-100 containing reverse micelles it was concluded that SDS promote substrate binding but do not change the rate of the catalytic reaction (Subramani *et al.,* 1996).

In the aqueous-organic two-phase system, organic solvents strongly influences the rate of enzymatic hydrolysis occurring at the interface. The great importance of the physical quality of the substrate surface can be admirably demonstrated in these assay systems. For instance, with cabbage PLD, almost no hydrolysis of PC could be observed with n-hexane as organic solvent whereas the hydrolysis was rapidly completed when n-hexane was either substituted with diethyl ether or a small amount of an aliphatic alcohol was added to n-hexane (Hirche *et al.,* 1997). In an extensive analysis of these solvent effects, it was proposed that the interfacial pressure in the reaction system reflecting the package density of the PC aggregates was the decisive parameter. The package density could be correlated with the enzyme activity which differed by the factor of 300 for the best (diethyl ether) and the worst (isooctane) solvent (Hirche and Ulbrich-Hofmann, 1999). The optimum interfacial pressures were in the same range as found in very early studies by film balance studies on PC monolayers at water air interfaces (Quarles and Dawson, 1969a; Demel *et al.,* 1975).

2.2.2.5 Inhibitors

Phosphate analogs such as aluminum fluoride (AlF_4^-) ions showed an inhibitory effect on PLD in cell-free lysates from rat submandibular gland (Li and Fleming, 1999a) as well as from cabbage (Li and Fleming, 1999b). From the experiments a competitive mechanism, in which AlF_4^- mimics the phosphate of the substrate with positive cooperativity among multiple binding sites was concluded (Li and Fleming, 1999b). Additionally, 1,3-diacylglycero-2-phosphocholines (1,3 PC), which are regioisomers of the natural phospholipid structures also

act as competitive inhibitors of plant PLDs as demonstrated for the enzyme isolated from cabbage leaves (Haftendorn *et al.,* 2000). All these compounds were not substrates for PLD. From kinetic experiments the existence of at least two substrate binding sites was derived, where one site binds substrate that functions as regulator without catalytic activity, while the other one is the catalytic site (Dittrich *et al.,* 1998).

2.2.2.6 Activation by PIP_2

PIP_2 is required by mammalian and yeast PLDs for activation. It is suggested that a PH as well as a PX domains possess by these PLDs are responsible for activation by PIP_2 as both of these domains have shown PIP_2 binding in many membrane binding proteins (Dinitto and Lambright, 2006; Seet and Hong, 2006). For plant PLDs also, PIP_2 has been shown to be an important specific activator molecule. Its presence is crucial for the activity of PLDβ, PLDγ (Pappan *et al.,* 1997/1998; Qin *et al.,* 2006) and PLDζ1 (Qin and Wang, 2002) from Arabidopsis. Although not necessary, it also stimulates the activity of PLDα (Qin *et al.,* 1997; Pappan *et al.,* 1999) and PLDδ (Wang and Wang, 2001). In addition to a PIP_2 binding site characterized by the motif (K/R)xxxx(K/R)x(K/R)(K/R) occurring in *Arabidopsis* PLDs within the C2 domain, a second PIP_2 binding site near the first HKD motif in PLDβ was localized by mutational studies (Zheng *et al.,* 2002). A PIP_2 binding site is also located in the C2 domain of the two α-type PLDs from cabbage (Schaeffner *et al.,* 2002). In addition to the shortened PIP_2-binding motif within the C2 domain (KxxxxRxR, residues 62–69), an inverted PIP_2 binding motif (RxRxxxK, residues 430–437) could be identified in the catalytic domain of α-type PLDs from poppy (Lerchner *et al.,* 2005). From kinetic studies on PLDδ (*A. thaliana*) it was concluded that PIP_2 promotes lipid substrate binding to the active site of the PLD molecule (Qin *et al.,* 2002). The activation by Ca^{2+} ions is linked with the activation by PIP_2 as evident from the extensive study on the PLD isoenzymes from *Arabidopsis thaliana.*

2.2.2.7 Substrate specificity and transphosphatidylation potential

PLD is capable of hydrolyzing broad range of phospholipid substrates including PC, PG, PE, PS, PI, Lyso PC, cardiolipin, and plasmalogens with the preference depending on the enzyme source and isoform (Pappan and Wang, 1999; Waite, 1999). In general PC, PG and PE are good substrates, whereas PS, PI, cardiolipin and plasmalogens are utilized much less efficiently (Heller, 1978). The PLDα studied in plant tissues showed activity towards more than one phospholipid substrate such as PLD isolated from castor bean endosperm hydrolyzed PC, PG and PE, but showed no detectable hydrolysis towards PI and PS (Dyer *et al.,* 1994).

Also, PLD from cultured soybean cells and germinating sunflower seeds had a much higher hydrolytic activity towards PC, PE and PG than PI and PS (Abousalham *et al.,* 1997). Recently, the recombinantly expressed PLD2 from cabbage also showed substrate specificity in the following preference order PC >PG >PE, when analyzed in PC-detergent mixed micellar system (Dippe and Ulbrich-Hofmann, 2009a). Interestingly, when studied in aqueous-organic two-phase reaction system, the recombinantly expressed isoenzymes from cabbage (PLDα1 and α2) and from poppy (PLDα1 and α2) displayed hydrolysis of all phospholipids (PC, PG, PE, PS and PI), with the exception of poppy PLD1, which could not cleave PS, though the order of preferences of substrate varied for each isoenzymes (Dippe and Ulbrich-Hofmann, 2009b). Besides, it was found that for each isoenzyme, PE is the better substrate than all other phospholipids used with the exception of cabbage PLD1 which showed slight preference of PC over PE. In addition to head groups, PLD also exhibits some selectivity for acyl composition of phospholipid substrate. Thus, PLDα from sunflower was most active towards PC composed of medium chain (C_6-C_{10}) fatty acids, when PC is presented in detergent micelles (Abousalham *et al.,* 1997). Moreover, this PLD showed a slight preference for PC containing unsaturated fatty acids over PC with saturated fatty acids. Recently, α-type PLD from soybean also showed a preference for acyl chains of medium size (10:0 and 12:0) as well 16:0 but displayed low level of activation when presented with PC consisting of either one or both unsaturated fatty acyl chains (Abdelkafi and Abousalham, 2011). Moreover, this PLD can also cleave water soluble soybean lysophosphosphatidylcholine, although with lower activity. Also, with respect to the *sn*-2 position of phosphatides, catalysis is stereospecific. This stereospecificity, however, is completely lost if the 2-acyl group in the substrate is replaced by an alkyl group (Bugaut *et al.,* 1985). Besides, alkyl phosphate esters such as the anti-tumor agent hexadecylphosphocholine are also accepted as substrates by PLD from cabbage (Dittrich *et al.,* 1996; 1998). On the other hand, PLDβ and γ also shown to catalyze the PC, PG and PE but their activities towards these phospholipids depend on the presence of PIP_2 and high amounts of PE. Additionally, PLDβ and -γ have the capability of hydrolyzing PS.

Beside, hydrolysis, the transphosphatidylation function of PLD is widely used in specific PLD assays (Morris *et al.,* 1997). In addition to the natural components of phospholipids (glycerol, ethanolamine, serine, inositides), a wide range of compounds can serve as phosphatidyl acceptors such as saccharides, nucleosides, peptides, arsenocholine, N-heterocyclic alcohols and aromatic compounds (Ulbrich-Hofmann 2000). In general, primary alcohols are better

substrates than secondary one, while no tertiary alcohol is known to be introduced (Ulbrich-Hofmann *et al.,* 2005). Besides, size of alcohol is another criterion for acceptance as head groups. In a comparative study carried out with α-type PLD2 from cabbage and PLD from *Streptomyces* sp. (type VII, Sigma) in the synthesis of new phospholipids with zwitterionic head groups, the acceptable size of the alcohol group was estimated to be 129 and 153 $Å^3$ (Dippe *et al.,* 2008). By site-directed saturation mutagenesis on PLD from *S. antibioticus* a tyrosine residue (Y385) was identified, which limits the size of the head group to be introduced by the enzyme and the replacement of this residue enabled phosphatidylinositol synthesis by the enzyme (Masayama *et al.,* 2008). Furthermore, it was observed for PLD-A from poppy seedlings that it can synthesize PE from PC by transphosphatidylation but cannot cleave it (Oblozinsky *et al.,* 2005). On the other hand, α-type PLD2 from cabbage is able to cleave PI but unable to synthesize it from PC (Dippe and Ulbrich-Hofmann, 2009b). From differences in the acceptance of polar head groups with respect to released or incorporated alcohols, it can be derived that the corresponding residues do not bind to the same loci in the active site. This conclusion is in accordance with the proposed reaction mechanism in which the phosphorus atom is attacked by the nucleophile from the side opposite to the leaving group (Leiros *et al.,* 2004). Moreover, plant isoenzymes also differ in their transphosphatidylation potential such as the two α-type PLDs from cabbage and the two α-type PLDs from poppy (Dippe and Ulbrich-Hofmann, 2009a; Lerchner *et al.,* 2005). In the poppy PLDs which differ by eleven amino acids only, two amino acid residues near the first HKD motif contribute to this difference (Lerchner *et al.,* 2005). It was concluded from the reaction mechanism proposed by Leiros et al. (2004) that the transphosphatidylation potential differing in the PLDs of different sources should depend on the chemical and spatial surrounding of the two HKD motifs. Finally, it has recently been demonstrated for PLDα2 from cabbage that the transphosphatidylation rate of PC with the natural L-serine is ten times higher than with its enantiomer D-serine (Dippe and Ulbrich-Homann, 2009a). In biocatalytic applications as well, PLD has an outstanding position because of its transphosphatidylation reaction (Ulbrich-Hofmann, 2000; Ulbrich-Hofmann *et al.,* 2005). For the biocatalytic efficiency, the transphosphatidylation rate (V_T) related to the hydrolytic rate (V_H), which is called the transphosphatidylation potential (V_T/V_H), or to the total transformation rate ($V_T + V_H$), which is called the transphosphatidylation selectivity [$V_T/(V_T + V_H)$], respectively, are decisive parameters. In addition to the acceptor alcohol and the reaction medium, these parameters mainly depend on the PLD source (Hirche and Ulbrich-Hofmann, 1999).

2.3 Biological functions of PLD

According to Wang (2000), the functions of PLD in the cell could be divided into three categories: (1) lipid degradation, (2) membrane remodeling and (3) regulation/signal transduction. The catabolic properties of PLD play an important role especially in the context of external stresses such as injury (Wang, 2000), water deficit (Frank *et al.*, 2000) or pathogens (Young *et al.*, 1996). In these cases, an increase in the PLD concentration in the tissues, correlate directly with the exposure to stress. The one way in which PLD achieve plant response to stresses is by mediating the action and production of stress-related hormones, abscisic acid (ABA) (Fan *et al.*, 1997; Zhang *et al.*, 2004), jasmonic acid (Wang *et al.*, 2000), and ethylene (Lee *et al.*, 1998). Lipid degradation processes occur in all stages of development. So increased levels of PLD activity were also observed in the process of senescence (Fan *et al.*, 1997) and found in meristametic tissues with prominent proliferation and membrane remodeling activities (Xu *et al*, 1997). PLD dependent membrane remodeling includes, in particular, the lipid biosynthesis and changes in membrane lipid composition (Li *et al.*, 2009).

Cell regulation includes the role of PLD in cell signaling, vesicular trafficking and cytoskeletal rearrangements. The function of the PLD in cell signaling or regulation is mainly derives from its hydrolytic product PA (Pappan and Wang, 1999). The second messenger PA is subject to strong fluctuations in response to environmental and developmental stimuli (Munnik, 2001). The mechanism by which PA can affect cellular functions, includes direct binding to target protein or altering the biophysical property of the membrane. There can be two outcomes of the binding of PA to proteins: modulation of target protein/enzyme activity and/or tethering of protein to membrane. On the other hand, the biophysical effect of PA on membrane can change the membrane curvature and integrity or it can affect membrane protein interaction (Li *et al.*, 2009).

In yeast, PLD is required for the late phases of meiosis and sporulation (Rose *et al.*, 1995); its function in these processes may result from a role in membrane traficking (Xie *et al.*, 1998). In mammalian cells, PLD function is important for various processes, including vesicular traficking, secretion, mitogenesis, oxidative burst, and cytoskeletal rearrangement (Liscovitch *et al.*, 2000). Activation of PLD produces messengers that in turn activate various enzymes such as protein kinases, lipid kinases, phosphatases, and phospholipases.

3. Materials

3.1. Phospholipid substrates

20:0/20:0 PC	Avanti Polar lipids, Alabaster, USA
8:0/8:0 PC	Sigma, St. Louis, MO, USA
10:0/10:0 PC	Sigma, St. Louis, MO, USA
12:0/12:0 PC	Fluka, Germany
14:0/14:0 PC	Sigma, St. Louis, MO, USA
16:0/16:0 PC	Lipoid, Ludwigshafen, Germany
18:0/18:0 PC	Lipoid, Ludwigshafen, Germany
1,3-PC	Synthesized in our lab
Soya-phosphatidic acid	Lipoid, Ludwigshafen, Germany
Soya-phosphatidylcholine	Lipoid, Ludwigshafen, Germany
Soya-phosphatidylethanolamine	Lipoid, Ludwigshafen, Germany
Soya-phosphatidylglycerol	Lipoid, Ludwigshafen, Germany
Soya-phosphatidylserine	Sigma, St. Louis, MO, USA
Soya-phosphatidyinositol	Avanti Polar lipids, Alabaster, USA
18:1/18:1 PC	Sigma, St. Louis MO, USA
16:0/18:1 PC	Sigma, St. Louis MO, USA

3.2. Chemicals

1-Butanol	Sigma, St. Louis MO, USA
1-Octanol	Fluka, Germany
2-Propanol	Merck, Darmstadt, Germany
1-Propanol	Merck, Darmstadt, Germany
2-Butanol	Sigma, St. Louis MO, USA
2-Hexanol	Sigma-Aldrich Steinheim, Germany
2-Octanol (distilled)	Sigma-Aldrich Steinheim, Germany
Aluminum chloride	Merck, Darmstadt, Germany
Benzylalcohol	VEB Laborchemie Apolda, Germany
BSA	Pierce, Rockford, USA
Brij 58	AppliChem Dermastadt, Germany
Calciumchloride, dihydrat	Merck, Darmstadt, Germany
Chloroform	Merck, Darmstadt, Germany
Choline oxidase	Sigma Aldrich, USA
Copper-II-sulphate, pentahydrate	Sigma, St. Louis MO, USA
CTAB	Roth, Kalsruhe, Germany
Dichloromethane	Sigma-Aldrich, Steinheim, Germany
D-Serine	VEB Berlin Chemie, Berlin, Germany
D-Sorbitol	Serva, Heidelberg, Germany
EDTA, disodiumsalt	AppliChem Dermastadt, Germany
Ethanol (96%)	Merck, Darmstadt, Germany
Ethanolamine (distilled)	Sigma-Aldrich, Steinheim, Germany
Ethylene glycol	VEB Laborchemie Apolda, Germany
Formic acid	Sigma, St. Louis MO, USA
Glycerol (99%) water free	Merck, Darmstadt, Germany

HEPES	AppliChem, Dermastadt, Germany
HPTLC plates	Merck, Darmstadt, Germany
Hydrochloric acid 37%	Roth, Karlsruhe, Germany
IEF markers	Serva, Heidelberg, Germany
Iron-(III)-chloride, hexahydrate	Merck, Darmstadt, Germany
Lithium chloride	Roth, Kalsruhe, Germany
L-Serine	Reanal, Budapest, Ungarn
Magnesium chloride	J.T. Baker, Holland
Manganese chloride	Roth, Kalsruhe, Germany
MES	AppliChem Dermastadt, Germany
Methanol 99.8% (v/v)	Roth, Kalsruhe, Germany
myo-Inositol	Sigma, St. Louis MO, USA
n-Hexane	VWR Prolabo, France
Nickel chloride	Roth, Kalsruhe, Germany
Phosphatidyl-*p*-nitrophenol	Synthesized in our lab
Phosphoric acid 85%, (w/v)	Roth, Kalsruhe, Germany
PIP_2	Avanti Polar lipids, Alabaster, USA
PIPES	Applichem, Darmstadt, Germany
Potassium chloride	J.T. Baker, Holland
Potassium permanganate	VEB Laborchemie Apolda, Germany
Silver nitrate	Roth, Kalsruhe, Germany
Sodium acetate, trihydrate	Merck, Darmstadt, Germany
Sodium chloride	Merck, Darmstadt, Germany
Sodium deoxycholate	Roth, Kalsruhe, Germany
Sodium dodecylsulphate	Roth, Kalsruhe, Germany
Sodium hydroxide	Merck, Darmstadt, Germany
Sodium orthovanadate	Sigma, St. Louis, MO, USA
Sodium salicylate	Sigma, St. Louis, MO, USA
Sodium fluoride	Sigma, St. Louis, MO, USA
Toluene	VWR Prolabo, France
Trypsin	Roche Diagnostics (Manheim, Germany)
TRIS	Roth, Karlsruhe, Germany
Triton X-100	Applichem, Darmstadt, Germany
Tween 20	Sigma, St. Louis, MO, USA
Zinc chloride	Merck, Darmstadt, Germany

3.3. Chromatographic material and mustard seeds

Octyl-Sepharose CL-4B	GE Health care, USA
Source-15Q	GE Health care, USA
Mustard seeds	Local market of Aligarh, India

3.4. Instruments

PHAST system	GE Healthcare, USA
Äkta-FPLC	GE Health care, USA
Transblot SD	BIO-RAD, Munich, Germany
Densitometer	Desaga, Heidelberg, Germany
HPTLC applicator	Desaga, Heidelberg, Germany
Horizontal chamber	Camag, Muttenz, Switzerland

Vibracell	Fisher Bioblock Scientific, France
Speed Vac Plus SC110 A	Savant Life Science International
JASCO V-660	JASCO, Germany
827 pHLab	Metrohm Swiss mode
Centrifuges Avanti 7301	Avanti, USA
Micro-plate reader HT-1	Biotek Instruments, Germany

4. Methods

4.1. Purification of PLD from mustard seeds

4.1.1. Preparation of delipidized powder

Dry mustard seeds (100 g) were grounded by mortar and pestle into a fine powder using liquid nitrogen. The powder was then suspended and mixed in 1 L of n-hexane on magnetic stirrer for overnight to remove the lipids. The powder was filtered and washed further with 1 L of n-hexane, and then left at room temperature for overnight to completely remove the solvent. This delipidized seed powder was kept at -20^{o}C until use. From 100 g mustard seeds, 60 g of delipidized powder was obtained.

4.1.2. Preparation of crude extract

60 g of delipidized powder (described above) was mixed and homogenized in 360 ml of 30 mM PIPES buffer, pH 7.0 containing 0.5 mM PMSF (phenylmethylsulfonyl fluoride), 10 mM potassium chloride and 1 mM EDTA using mortar and pestle. The suspension was filtered through two layers of cheese cloth (which was prewashed and dried) and the filtrate was centrifuged at 30,000 *g* for 1 hour. The pellet was discarded and the supernatant obtained (300 ml) was regarded as crude extract. All extraction steps were performed at 4^{o}C. The protein concentration and PLD activity in crude extract were analyzed by Bradford (section 4.2.) and iron (III) complexation assay (section 4.9.1.1) respectively.

4.1.3. Ammonium sulphate precipitation

To 300 ml of crude extract (obtained in section 4.1.2), solid ammonium sulphate was added so as to get 25% saturated solution. The solution was left overnight for equilibration and then centrifuged at 30,000 *g* for 40 min. The precipitate containing only a negligible amount of PLD activity was discarded. To the supernatant obtained, solid ammonium sulphate was again added to reach 50% saturation. Again, this 50% saturated solution was left overnight for equilibration and then centrifuged at 30,000 *g* for 40 min. The supernatant was discarded as it contains negligible amount of PLD activity and the precipitate obtained was re-solubilised in 30 mM PIPES buffer, pH 7.0 containing 0.5 mM PMSF, 10 mM KCl and 1 mM EDTA. The resulting solution was dialyzed extensively against 2 L of the same buffer solution (using ServaPOR, 8 kDa cut off range dialysis bag) for 8 hours with two buffer changes. However in the second buffer exchange, EDTA was omitted as the next purification step involved hydrophobic interaction chromatography mediated by calcium ions. Some precipitation was observed during dialysis, therefore the sample was centrifuged at 6000 *g* for 10 min so as to

get a clear solution. All the steps were performed at 4^{o}C. Finally, the clear solution (120 ml) was divided into four aliquots of 30 ml and stored in 50 ml falcon tubes at -20^{o}C. After each step of the ammonium sulphate precipitation, the protein content and PLD activity in the precipitate as well as in the supernatant were analyzed by Bradford (section 4.2) and iron (III) complexation assay (section 4.9.1.1) respectively.

4.1.4. Calcium mediated hydrophobic interaction chromatography

A 30 ml aliquot of the solution obtained in 4.1.3 was withdrawn from -20^{o}C and thawed at room temperature. $CaCl_2$ was added to the above sample to yield a final concentration of 100 mM. The solution was centrifuged at 6000 *g* for 5 min at 4^{o}C to remove any visible precipitate formed after addition of $CaCl_2$. The clear solution was then loaded onto 30 ml of Octyl-Sepharose CL-4B matrix (GE Healthcare) which was packed in a column and was pre-equilibrated with 30 mM PIPES buffer, pH 7.0 containing 100 mM $CaCl_2$. The binding of enzyme was carried out in the same buffer at the flow rate of 0.5 ml per minute. The unbound proteins were removed by washing the column with the same buffer at the flow rate of 1 ml per minute. Finally the elution of bound proteins was performed using 5 mM PIPES buffer, pH 7.0 containing 0.1 mM EDTA at a flow rate of 1 ml per minute. Fractions of 5 ml were collected. This operation was performed using a peristaltic pump (Pharmacia, USA) attached to a fraction collector. The whole process was carried out at room temperature which is critical for binding of PLD activity from mustard seeds on the Octyl-Sepharose CL-4B matrix. The eluted fractions possessing PLD activity were pooled and kept at -20^{o}C. The protein content and PLD activity in each eluted fraction was analyzed by recording the absorbance at 280 nm and iron (III) complexation assay (section 4.9.1.1.), respectively.

The active fractions obtained after four such cycles of purification were pooled together so as to obtain a substantial amount of PLD enzyme before performing the next step of purification.

4.1.5. Anion exchange chromatography

Anion exchange chromatography was performed using Source 15Q matrix (GE Healthcare) on an Äkta FPLC system (GE Healthcare) at 4°C. The pooled sample (a total of 80 ml) obtained in section 4.1.4 was loaded onto 3.6 ml of matrix (packed in a column) which was pre-equilibrated with 10 mM PIPES buffer, pH 7.0. The binding and washing of unbound proteins were executed in the same buffer at a flow rate of 1 ml per minute. Finally, the elution of bound proteins was carried out by a gradient of 0.1-0.75 M sodium chloride (NaCl) in 10 mM PIPES buffer, pH 7.0. Fractions of 2 ml were collected. The PLD activity in each

fraction was assessed by the iron (III) complexation assay (section 4.9.1.1). The active fractions containing PLD activity were analyzed for their purity with SDS-PAGE (10% crosslinking) (according to section 4.3) and those displaying a single band in SDS-PAGE were pooled and subsequently dialyzed in 2 L of 10 mM PIPES buffer, pH 7.0 for 8 hours at 4^oC with 2 buffer exchanges. The dialyzed sample was stored at -20^oC for further PLD characterization studies.

4.2. Determination of protein concentration

The protein content in crude extracts and in samples after ammonium sulphate precipitation was determined by Bradford (1976) with BSA as standard while the protein concentration in pooled and active samples after hydrophobic interaction chromatography on Octyl-Sepharose CL-4B and anion exchange chromatography on Source 15Q was quantified by Micro BCA protein assay kit (Rockford, USA) according to manufacturer instructions, using BSA as a standard.

4.3. SDS-PAGE and molecular mass determination

The denaturing PAGE in the presence of sodium dodecylsulphate (SDS-PAGE) was performed as described by Laemmili (1970). Proteins were resolved on 10% resolving and 4% stacking slab SDS- minigels (0.75 mm thick). Protein samples were mixed in loading buffer (2 % (w/v) bromophenol blue, 2 mM SDS, 0.1 mM β-mercaptoethanol, 0.1 mM EDTA) in the ratio of 1:2 and heated at 90^oC for 2 min. The gels were run in running buffer (25 mM Tris, 200 mM glycine and 0.5 mM SDS) at a constant voltage of 200 V. The gel was stained with silver as described by Blum *et.al,* 1987.

The molecular mass of mustard PLD was determined by performing a SDS-PAGE (as described above) of purified mustard PLD (obtained in section 4.1.5) along with pre-stained protein markers with the molecular mass ranging from 10 kDa to 250 kDa (PageRuler from Fermentas Life Sciences). The mobility of the protein bands relative to the dye front were plotted against the logarithm of the molecular masses of marker proteins. The molecular mass of PLD was then calculated by the linear regression analysis from the data obtained by the plot.

4.4. Immunoblotting

Purified mustard PLD (obtained in section 4.1.5) was electrophoresed by SDS-PAGE (10% gels) along with purified PLD1 from poppy (Lerchner *et al.*, 2005) and pre-stained protein markers (PageRuler from Fermentas Life Sciences) as described by Laemmli (1970). After electrophoresis, the transfer of protein from the gel onto nitrocellulose membrane was performed into a transfer buffer (25 mM Tris, 200 mM glycine and 20% (v/v) methanol). The transfer was carried out for 2 hours at the rate of 2.5 mA/cm^2 of the gel area using a Trans-Blot SD apparatus (Bio-Rad). On completion of transfer, the membrane was washed with Tris-Buffer-Saline-Tween (TBST) (25 mM Tris, 100 mM NaCl, 0.05% (w/v) Tween-20) by 3 washes for 5 min each. The non-specific protein sites were blocked by incubating the membrane overnight at 4^oC in a blocking solution containing 4 % (w/v) skimmed milk in Tris-Buffer-Saline (TBS) (25 mM Tris, 100 mM NaCl). The membrane was again washed with TBST in similar manner as described above. The membrane was then incubated for 2 h with anti-poppy PLD1 antibodies (the polyclonal antibodies generated against α-PLD1 from poppy) (Oblozinsky *et al.*, 2011) in TBS containing 2 % (w/v) skimmed milk at a dilution of 1:20. The unbound primary antibodies were removed by washing with TBST as described above. Again, the non-specific protein sites were blocked by incubating the membrane for 20 min in TBS containing 2% (w/v) skimmed milk. Finally, the membrane was incubated with peroxidase-conjugated goat anti-rabbit Ig G (1:5000 dilutions) in TBS containing 0.5% (w/v) skimmed milk for 80 min. Then the membrane was washed by 3 washes (5 min each) in TBST. The bands were revealed with enhanced chemiluminiscence (ECL) Western blotting solutions (Amersham, USA) following the instructions of manufacturer in a dark room. All the immunoblotting procedures were carried out at room temperature except stated otherwise.

4.5. Non-denaturing polyacrylamide gel electrophoresis (NATIVE-PAGE)

The electrophoretic separation of the PLD was performed under non-denaturing conditions on a 10% resolving gel according to Novatna *et al.* (1999). Enzyme solution (30 µl) containing 1.56 µg of PLD was mixed with equal volume of sample buffer (0.5 g bromophenol blue and 10% glycerol). Increasing volumes of sample (4-20µl) containing 0.1-0.52 µg of PLD were then loaded onto the gel. The electrophoresis was carried out in running buffer (25 mM glycine and 80 mM Tris, pH 8.3) without SDS at 6^oC with increasing voltage up to 200 V, to get a constant current of 6-8 mA. The gel was stained with silver (Blum *et.al.*, 1987).

4.6. Native blotting and activity staining

For native blotting and activity staining, a NATIVE-PAGE of the PLD was performed as explained in section 4.5. The transfer of protein from the gel onto the nitrocellulose membrane was then performed under non-denaturing conditions (without methanol) using a discontinuous buffer system (anode buffer I: 0.3 M Tris (pH 10.8); anode buffer II: 25 mM Tris; cathode buffer: 40 mM ε-aminocaproic acid, 0.01% (w/v) SDS (~ pH 7.9) for 30 min at the rate of 5 mA per cm^2 of the gel area (Kyhse-Andersen, 1984) at 6^oC. After the transfer, the membrane was rinsed with distilled water and was put in a plane bowl. The membrane was then incubated for 30 min at room temperature with 1 ml of substrate solution (10 mM SPC in 0.2 M sodium acetate buffer, pH 5.5 containing 0.1 M $CaCl_2$ which was vortexed and sonicated). Then the membrane was covered carefully with 2 ml of chromogenic mixture consisting of 10 mM Tris-HCl pH 8.0, 5 mM aminoantipyrene, 8 mM phenol, 3.0 U/ml choline oxidase and 0.2 U/ml peroxidase for 2 h without any movement or shaking.

4.7. Isoelectric focussing

To determine the isoelectric point of PLD, isoelectric focussing using Servalyt-Precotes 3-10 was conducted using a PHAST electrophoresis system (Pharmacia, USA). The IEF markers, pH 3-10 from Serva were used as standard. The focussing took 10 min after the end voltage was achieved. The gel was stained with silver as described by Blum *et al.*, 1987.

4.8. Mass spectrometry

The SDS-PAGE was carried out of an extensively dialyzed sample containing 5.2µg of purified PLD according to section 4.3. The gel was stained with Coomassie brilliant blue G-250 solution (0.25% w/v Coomassie brilliant blue G 250 dye in 50% v/v ethanol and 10% v/v acetic acid) for overnight and subsequently de-stained with a solution consisted of ethanol, glacial acetic acid and distilled water in a ratio of 2: 1: 2.

After destaining, the protein bands were cut out of the gel, washed three times with water, twice with 10 mM ammonium bicarbonate and finally with 10 mM ammonium bicarbonate in 50% acetonitrile. The gel pieces were dried and reswollen in 20 µl of 10mM ammonium bicarbonate (pH 8.0) and digested with 1µg trypsin (Promega, Madison, WI, USA) overnight at 37°C. The tryptic peptides were then, extracted from the gel pieces and injected into a CapLC (Micromass, Manchester, UK) equipped with an autosampler, gradient and auxillary pump. 4µl were injected via “microliter pickup” mode and desalted on-line through a

350µm×5mm Symmetry300™ C_{18} trap column (Waters, USA). The peptides were separated on a 75 µm ×15 cm, 3µm dC18 Atlantis NanoEase™ column (Waters, USA) using a gradient of 5-60% solution A for 30 min. Then the ratio was changed within 10 min to 95% B. This ratio was maintained for 4 min before returning to the initial conditions. (where, A: 5% (v/v) acetonitrile/95% 0.1% (v/v) formic acid and B: 95% (v/v) acetonitrile/5% 0.1% (v/v) formic acid)]. Then, MS/MS experiments were performed on a Q-TOF 2 mass spectrometer (Micromass, Manchester, UK). The mass spectrometer is equipped with a modified nano-ESI source to hold a pico-tip (New Objective, Cambridge, MA, USA). The collision energy was determined on the fly based on the mass and charge state of the peptide. Charge state recognition was used to switch into MS/MS mode on only doubly and triply charged ions. Three components were monitored per survey scan. Spectra were acquired in MS mode at 1s/scan and in MS/MS mode at 3s/scan.

The data were processed to generate a searchable peak list. The files were fed into the search engine MASCOT (Matrix Science, London, UK) (*http://www.matrixscience.com*). The data were searched against the MSDB protein sequence database.

The mass spectrometry measurements were carried out by Dr. Angelika Scheirhorn, Institute of Biotechnology and Biochemistry, Martin-Luther University, Halle-Wittenberg.

4.9. Determination of the catalytic properties of the PLD in mixed micellar system

4.9.1. PLD activity assays

4.9.1.1. Iron (III) complexation assay

For the standard determination of PLD activity, hydrolysis of phospholipid substrate was measured in a mixed micellar system by the iron (III) complexation method (Dippe and Ulbrich-Hofmann, 2009a) carried out in microtitre plates. The assay was based on the quantitative determination of PA (released by the hydrolytic activity of PLD on phospholipid substrate), which in turn depends on the binding of Fe^{3+} ions to PA in competition with its binding to salicylate in the coloured iron (III)-salicylate complex (fig 4.1). Precisely, the iron (III)-salicylate complex is a purple coloured complex with the maximum absorption at 500 nm. The binding of a negatively charged PA to Fe^{3+} ions of this complex results in the decrease of its purple colour, which is measured at 490 nm and this corresponds directly to the amount of PA released in the reaction.

The standard assay was carried out with PC as substrate. 3 mg (0.035 mmoles) PC from soya was dissolved in chloroform and the solution was evaporated overnight to dryness at room temperature. After the addition of 0.0175 mmoles of SDS and 0.06 mmoles of Triton X-100, the solution was vortexed and then ultrasonicated for 2 min using a probe sonicator (800 watts/second), followed by centrifugation for 2 min at 10,000 *g*. This substrate stock solution has a PC: SDS: Triton X-100 in a molar ratio of 1:0.5:1.6. In the following, this solution will be designated as PC solution.

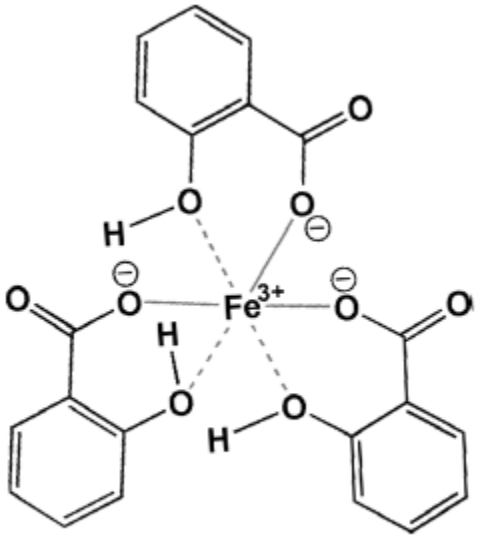

Fig 4.1. Iron (III) salicylate complex (FeSal)

The standard reaction mixture of 0.05 ml contained 10 µl of PC solution, 30µl of appropriately diluted enzyme solution and 10 µl of 350 mM $CaCl_2$ in 0.5 M MES-NaOH, pH 5.5 buffer. The reaction was performed at 37^{o}C for the appropriate time (within the linear part of the progress curve) and was arrested by adding 125 µl of stopping solution (1 mM $FeCl_3$, 6 mM sodium salicylate and 10 mM Triton X-100 in 500 mM HCOOH/ NaOH, pH 4.0). The stopping solution will be stated briefly as FeSal solution. The absorbance was measured at 490 nm after equilibration for 5 min. All the reactions were carried out in duplicates. The amount of PA formed during the reaction was calculated by the calibration curves obtained with PC/PA mixtures (fig 4.2) as described by Dippe and Ulbrich-Hofmann, 2009a. One unit of enzyme is defined as the amount of enzyme which releases 1 µmol of PA per min.

For analyzing the substrate specificity of PLD for PG and PE, the standard curves were obtained for corresponding phospholipid in the similar manner as done for PC.

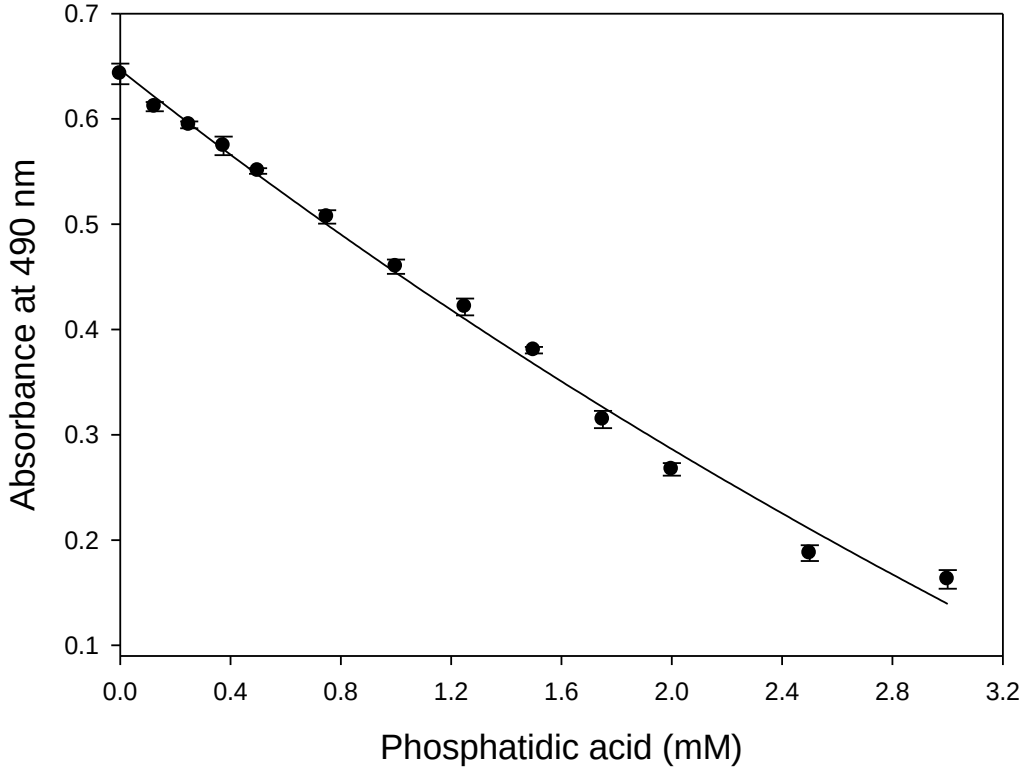

Fig. 4.2. Standard curve for the determination of PA by FeSal in the presence of PC. Mixture of PC and PA (in a total concentration of 5 mM) both from soyabean, were incubated with FeSal solution for 5 min and subsequently analyzed at 490 nm as described in section 4.9.1.1. Data are the means ± standard deviations obtained from three independent measurements.

4.9.1.2. Phosphatidyl-*p*-nitrophenol assay

This method involves the hydrolysis of phophatidyl-*p*-nitrophenol (P*p*NP) (synthesized in our lab as described by D'Arrigo *et al.* 1995) to release *p*-nitrophenol as a hydrolytic product which is measured at 405 nm. The typical reaction mixture (0.2 ml), consisted of 20µl of substrate solution (10 mM PpNP, 10% (v/v) Triton X-100 and 10 mM SDS), 130µl of 0.154 M sodium acetate buffer, 0.108 M $CaCl_2$, pH 5.5 and 50 µl of appropriately diluted enzyme solution. After incubation for 15 min (which is within the linear part of the progress curve) at 37^oC, the reaction was stopped by addition of 50 µl 1 M Tris/HCl, pH 8.0, containing 0.1 M EDTA. The reaction was carried out in duplicates. The amount of product formed during reaction was calculated by the calibration curves obtained with *p*-nitophenol (extinction coefficient of *p*-nitrophenol at 405 nm is 18450 M^{-1} cm^{-1}). One unit of enzyme is defined as the amount of enzyme which releases 1 µmol of *p*-nitrophenol per min.

4.9.2. Determination of kinetic parameters

For the determination of kinetic parameters of PLD with PC as substrate, the initial rates of reaction as a function of substrate concentrations (0-5 mM) were measured by standard assay procedure (section 4.9.1.1). The initial rate of hydrolysis was plotted as a function of substrate concentration. Kinetic data were fitted with the well-known Michaelis-Menten equation:

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

where v and V_{max} are the observed initial and the maximum rate, [S] is the concentration of substrate (PC) and K_m is a Michaelis constant characterizing binding affinities. The fitting of the data was performed using the SigmaPlot version 11.0 (Jandel Sceintific). All the experiments were performed in duplicates.

4.9.3. Determination of pH optimum and stability

The pH optimum of PLD was determined (with PC as substrate) by the standard activity assay as described in section 4.9.1.1 except that the buffers used in the reaction mixture were 100 mM sodium-formate (pH 3.0-3.5), 100 mM sodium acetate (pH 4.0-5.5), 100 mM MES (pH 5.5-6.5), 100 mM HEPES (pH 6.5-7.0) and 100 mM Tris-HCl (pH 7.5-9.0) all containing 70 mM calcium chloride. All experiments were performed in duplicate.

The pH stability of PLD was studied by incubating the enzyme in the buffers of different pH at 25^{o}C for 1.5 hours and then measuring the residual activity by standard activity assay at pH 5.5 (as described in section 4.9.1.1). The buffers used were 50 mM sodium-acetate (pH 4.5-6.0), 50 mM MES (pH 5.5-6.0), 50 mM PIPES (pH 6.5-7.0), 50 mM HEPES (pH 7.5-8.0) and 50 mM glycine (8.5-10.0). All experiments were performed in duplicate.

4.9.4. Determination of temperature optimum and thermal stability

The temperature optimum of mustard PLD was assessed (with PC as substrate) by measuring the conversion rate at temperatures ranging from 25^{o}C to 70^{o}C. Reaction mixture (0.05 ml) consisting of PLD, substrate PC solution and buffer (100 mM MES-NaOH, pH 5.5 and 70 mM $CaCl_2$) was incubated for 6 min at temperatures ranging from 25^{o}C-70^{o}C (as described in section 4.9.1.1). The activity of PLD was defined as the released amount of PA per min and related to the activity at 37^{o}C. The activity values were plotted as a function of temperature.

According to the Arrhenius equation:

$$k = Ae^{-E_a/RT}$$

where, k is the rate constant,

A is known as the pre-exponential factor,

R is the gas constant,

T is the absolute temperature,

E_a is the activation energy of the reaction.

a plot of natural log (ln) of velocity (relative activity) against the reciprocal of temperature (1/T) in Kelvin gives a straight line (in the temperature range of 298 to 318 K). From the slope of straight line, the activation energy of enzyme was calculated (Price and Stevens, 1982)

The thermal stability of PLD was measured by incubating the enzyme at different temperatures (25^{o}C -70^{o}C) for 8 min in 0.05 M PIPES, pH 7.0 followed by immediate chilling on ice for 3 min. After keeping the enzyme at room temperature for 2 min, the residual activity of PLD was measured by the standard activity assay at 37^{o}C and pH 5.5 (as described in section 4.9.1.1).

4.9.5. Determination of the effect of various metal ions on activity and stability of the PLD

The effect of various metal ions (Ca^{2+}, Mn^{2+}, Mg^{2+}, Ni^{3+}, Co^{2+} and Li^{2+}) on PLD activity was analyzed with the standard activity assay (section 4.9.1.1) except that the reaction buffer (0.1 M MES, pH 5.5) contained the corresponding metal chlorides (100 mM). Blanks were prepared for respective metal ions so as to assess any interference of the metal ions with the reaction system itself.

The effect of metal ions ($CaCl_2$ and NaCl) on the stability of PLD was studied by incubating the enzyme in two salt solutions of same ionic strength i.e. $CaCl_2$ (40 mM) and NaCl solution (120 mM) (prepared in 0.05 M PIPES, pH 7.0) at room temperature for different intervals of time (15-90 min). The residual activity was measured by the standard activity assay at 37^{o}C and pH 5.5 (as described in section 4.9.1.1).

4.9.6. Determination of the PLD activity at different concentrations of Ca^{2+} ions and different pH values

The effect of calcium ions on the hydrolytic activity of PLD was investigated by standard assay (section 4.9.1.1) except that the reaction buffer (0.1 M MES, pH 5.5) contained different concentrations of calcium chloride (0-200 mM). For each $CaCl_2$ concentration the ionic strength was adjusted by equivalent amount of NaCl (0-600 mM).

The effect of calcium ions as a function of pH on the activity of PLD was determined by standard assay procedure (section 4.9.1.1) except that the buffers, 0.1 M sodium acetate (pH 4.5-5.0), 0.1 M MES (pH 5.5-6.0) and 0.1 M HEPES (pH 6.5-7.5) containing different concentrations of $CaCl_2$ (0-75 mM) were used.

4.9.7. Determination of the effect of substrate structure on the PLD activity

The influence of the chemical structure of the substrate phospholipid on the PLD activity was studied by the standard assay as described in section 4.9.1.1 but using substrate with different alcoholic head groups PG, PE and PC or different fatty acyl chains (8:0/8:0 PC, 10:0/10:0 PC, 12:0/12:0 PC, 14:0/14:0 PC, 16:0/16:0 PC, 18:0/18:0 PC, 20:0/20:0 PC, 16:0/18:1 PC and 18:1/18:1 PC) in their *sn*-1 and *sn*-2 positions, respectively. Furthermore, the effect of the position of fatty acyl chains in the phospholipid glycerol backbone was studied with PC molecular species having fatty acyl chains of various lengths (8:0/8:0, 12:0/12:0 and 14:0/C14:0) in their *sn*-1 and *sn*-3 positions, respectively. These PC molecular species, referred to as 1,3-PC, were synthesized in our laboratory (Haftendorn and Ulbrich-Hofmann, 1995). In each case, the substrate solutions were prepared by mixing respective phospholipid substrate (5 mM) with detergents in the molar ratio of (phospholipid: SDS: Triton X-100) 1:0.5:1.6 as used in standard assay protocol (section 4.9.1.1).

4.9.8. Determination of the effect of detergents on the PLD activity

The influence of various detergents (SDS, sodium deoxycholate, Triton X-100, Tween 20, Brij 58 and CTAB) on the activity of PLD was studied in analogy to the standard assay procedure as illustrated in section 4.9.1.1. The substrate solutions having different molar ratios of PC to detergents were prepared by mixing PC (5 mM) with different amounts of respective detergents followed by co-sonification and centrifugation at 10,000 *g* for 2 min (as explained in section 4.9.1.1). Substrate solution without detergent was also prepared in the same manner except that water was used instead of any detergents. Formation of precipitates

were observed during the reaction when anionic detergents i.e. SDS and sodium deoxycholate were used for the study. Therefore, a fixed amount of Triton X-100 (40 mM) was added along with these anionic detergents in the substrate solution.

The calibration curves for each PC to detergent ratio were prepared separately to exclude an interference of the individual detergents in the reaction procedure. All the reactions were performed in duplicates.

4.9.9. Determination of the effect of Inhibitors and PIP_2 on the PLD activity

The influence of the inhibitors, aluminum fluoride (AlF_4^-) (a combination of sodium fluoride (NaF) and aluminium chloride ($AlCl_3$)) and sodium orthovanadate (Na_3VO_4) on the activity of PLD was studied by the P*p*NP assay (section 4.9.1.2). Briefly, appropriate amounts of purified PLD were incubated and mixed with Na_3VO_4 (0-400 µM) and AlF_4^- (0-100 µM) in total volumes of 0.24 ml adjusted with PIPES buffer, pH 7.0. After 5 min of incubation, samples (50 µl) were withdrawn from the incubation mixture and added to the reaction medium consisting of 130 µl of 0.154 M sodium acetate, pH 5.5 containing 0.108 M $CaCl_2$ and 20µl P*p*NP substrate (already placed in the wells of the microtitre plates). The rest of the reactions were performed as described in section 4.9.1.2. The concentration of inhibitor at which the enzyme activity reduced to 50% (IC_{50}) was calculated using a sigmoidal model for the ligand-binding dose response curve (SigmaPlot software, version 11.0).

The effect of PIP_2 as a function of calcium ions concentration on the activity of the PLD was investigated by the standard assay (described in section 4.9.1.1). The substrate solution was prepared by co-sonicating PIP_2 with PC in the presence of SDS and Triton X-100 so as to have PC : SDS : Triton X-100 in the molar ratio of 1:0.5: 1.6 The percent molar ratio of PC to PIP_2 used in the final reaction mixture was 98:2 (i.e. 4.9 mM PC + 0.1 mM PIP_2). The effect of neomycin on the PIP_2 dependent hydrolytic activity was studied by using additionally 1mM neomycin in the reaction mixture.

4.10. Determination of the catalytic properties of the PLD in aqueous-organic two-phase system

4.10.1. PLD activity assay

The hydrolytic and transphosphatidylation activities in two-phase system were determined according to Hirche *et al.*, (1997). In this assay system, the reaction mixture consisted of an

organic phase containing the substrate phospholipid and the aqueous phase containing the enzyme in buffer system.

The standard hydrolytic reaction was performed by placing the organic phase (620 µl of n-hexane and 2-octanol (0.43 mmol) containing 1.3 µmol PC) in 1.5 ml screw flasks which were sealed by Teflon-silicon septa. The reaction was started by adding 80 µl of aqueous phase containing 15 mU of PLD (as determined by the standard assay according to section 4.9.1.1) in 300 mM sodium acetate buffer/120 mM $CaCl_2$ (pH 5.5). In case of transphosphatidylation reaction, the aqueous phase additionally contained 3 M acceptor alcohol. The reaction was carried out on a horizontal shaker at 30°C and with a speed of 400 rpm. During the reaction, aliquots of 15 µl were withdrawn from the organic phase and 1.5 µl of the sample was applied to HPTLC plate silica gel 60 (Merck, Darmstadt, Germany) by the TLC applicator AS 30 (Desaga, Heidelberg, Germany). The aqueous phase did not contain detectable amounts of phospholipids. Development of the plate was performed by a solvent system comprising of chloroform: methanol: 25% ammonia (7:4:1, by volume) in a horizontal chamber (Camag, Muttenz, Switzerland) using the sandwich configuration at a relative humidity of 30-40%. The plate was then dried by heating at 100^{o}C for 5 min and the phospholipids were stained by carefully dipping the plate in a solution of 0.63 M $CuSO_4$ in 0.85 M H_3PO_4 followed by heating at 140^{o}C according to the method of Touchstone et al. (1983). The intensity of the phospholipid bands were quantified by densitometry at 560 nm with CD 60 densitometer (Desaga) according to Aurich *et al.* (1999).

The phospholipid contents of the sample were quantified by use of standard curves for the corresponding phospholipids (PC, PG, PE, PI, PS and PA) as explained by Aurich *et al.,* (1999). The initial rates of hydrolysis and transphosphatidylation were calculated from the increase of product formation within the linear range of the progress curve using Sigma software version 11.0. All the experiments were carried out in duplicates.

4.10.2. Determination of the effect of organic solvents on the PLD activity

The effect of organic solvents on the hydrolytic activity of PLD was studied by standard assay described in section 4.10.1. Precisely, PC (1.3 µmol) dissolved in different organic solvents like diethyl ether, chloroform, toluene, methylene dichloride and n-hexane/2-octanol (7.8:1) was used as organic phase. For solvents which are highly volatile (diethyl ether, chloroform and methylene chloride), a slight modification in the application of sample to the silica plate

was made i.e. after specified reaction time, samples of organic phase (15 µl) were withdrawn and were air dried. The residuals were dissolved in 15 µl of toluene, mixed and vortexed. The samples were then applied on to HPTLC silica plates by TLC applicator and analyzed as described in section 4.10.1. All the reactions were performed in duplicates.

The effect of aliphatic alcohols as additives in n-hexane/alcohol system was studied by the standard hydrolytic activity assay as described in section 4.10.1 except that varying amount of aliphatic alcohols (0-90 µl) and n-hexane (containing 1.3 µmol of PC) were used as organic phase. All the reactions were carried out in duplicates.

4.10.3. Determination of pH optimum

Effect of pH on the hydrolytic activity of PLD was determined by the activity assay as described in section 4.10.1 except that the 300 mM sodium acetate buffers, in the pH range 4.25-6.0, containing 120 mM $CaCl_2$ were used in the aqueous phase of the reaction mixture. All the reactions were carried out in duplicates.

4.10.4. Determination of the effect of Ca^{2+} ions concentration on the PLD activity

Effect of calcium ions on the hydrolytic activity of PLD was determined by the activity assay as described in section 4.10.1 except that the 300 mM sodium acetate buffer, pH 5.5 containing 0-120 mM $CaCl_2$ were used in the aqueous phase of the reaction mixture. The value of apparent binding constant (K*ca*) was calculated by fitting the data with single hyperbolic plot using SigmaPlot software (version 11.0). All the reactions were carried out in duplicates.

4.10.5. Determination of kinetic parameters

For the determination of kinetic parameters of PLD on PC as substrate in aqueous-organic two-phase system, the rate of reaction for a range of substrate concentration (0-3 mM) was measured as described in section 4.10.1. Fitting of the data was performed with Michaelis-Menten equation and the apparent Michaelis constant (K_m) and maximum velocity (V_{max}) values were calculated using the software SigmaPlot (version 11.0). All the reactions were carried out in duplicates.

4.10.6. Determination of the substrate specificity of the PLD

The substrate specificity of the PLD with naturally occurring phospholipids i.e. PE, PC, PG, PS and PI (all from soya) was studied with the hydrolytic activity assay as described in section 4.10.1, except that the organic phase consisting of the individual phospholipids in n-hexane/2-octanol (7.8:1) solvent system was used. All the reactions were performed in duplicates.

5. Results

5.1. Purification of PLD from mustard seeds

PLD was produced from mature Indian black mustard seeds. Dry mustard seeds were converted into a delipidized powder from which a PLD containing crude extract was prepared by homogenization, filtration and centrifugation. From the obtained crude extract, the enzyme could be purified using a three step procedure involving ammonium sulphate precipitation, calcium-mediated hydrophobic interaction chromatography on Octyl-Sepharose CL-4B and anion exchange chromatography on Source 15Q (as described in section 4.1).

One of the most successful example that resulted in the homogenous preparation of mustard PLD is demonstrated in the following. Fig 5.1 depicts the elution profile of mustard PLD on Octyl-Sepharose CL-4B. Fractions 1-22 (up to 220 ml) of elution profile showed unbound proteins that got eluted from the column with the equilibration buffer 30 mM PIPES, pH 7.0 containing 0.1 M $CaCl_2$ (described in section 4.1.4). The elution of protein was carried out from fraction 23 to 40 (total of 85 ml) with elution buffer 30 mM PIPES, pH 7.0 containing 0.1 mM EDTA. Fractions 28 to 31 (total of 20 ml) displaying highest PLD activity were pooled and stored at -20^{o}C for the anion exchange chromatography. This step resulted in a great rise of PLD specific activity (i.e. 104 µmol min^{-1} mg^{-1}) with the purification factor of more than 950 (table 5.1). The substantial amount of enzyme was produced by performing four such consecutive cycles of calcium mediated hydrophobic interaction chromatography (fig 5.1) and was subsequently applied for the next step of anion exchange chromatography on Source 15Q (illustrated in section 4.1.5). Fig 5.2 shows the elution profile of mustard PLD on Source 15Q column. Elution of PLD was carried out using a linear gradient of 0.1-0.75 M NaCl in 10 mM PIPES, pH 7.0. PLD activity began to appear in the effluents when the sodium chloride concentration approaches to 180 mM and continued till approximately 280 mM (elution volume 42-54 ml) (fig 5.2). Two fractions (corresponding to elution volume 44-47 ml) having the highest PLD activity and purity (as analyzed by SDS-PAGE) were pooled and dialyzed. PLD with a purification factor of more than 2000 and a yield of 14% was obtained after the final purification step of anion exchange chromatography (table 5.1).

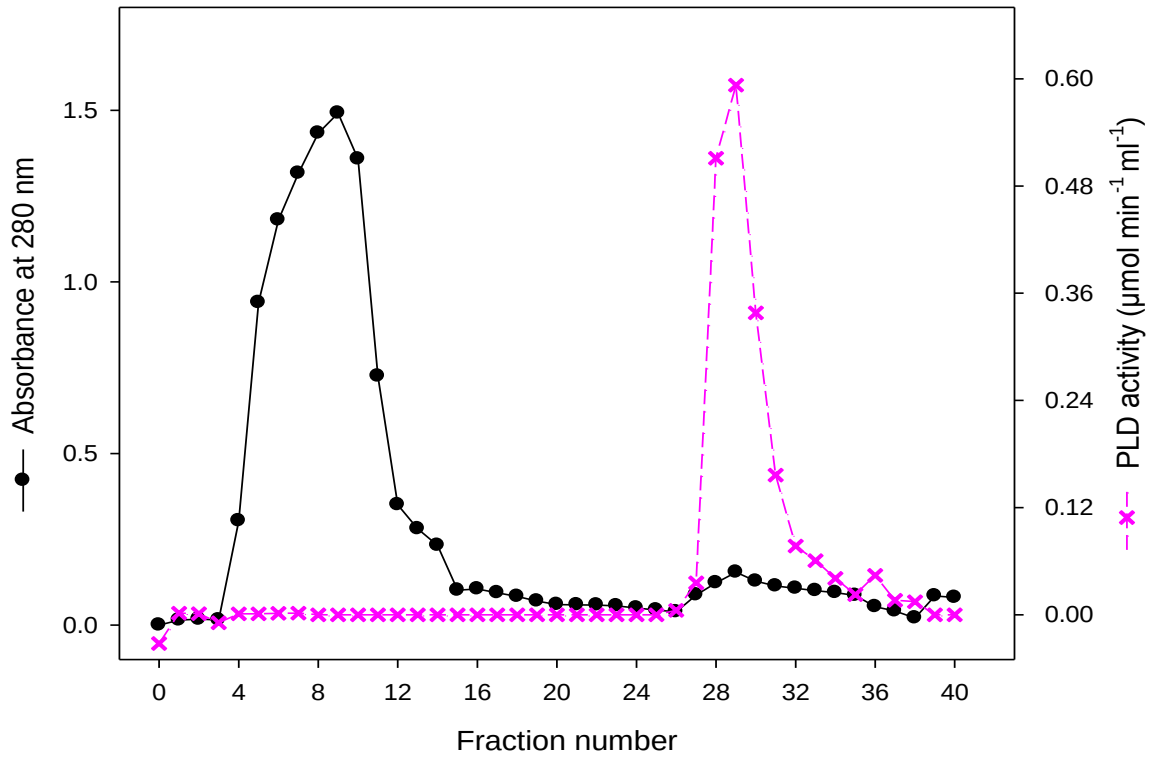

Fig. 5.1. **Elution profile of mustard PLD on Octyl-sepharose CL-4B column.** 30 ml of dialyzed sample after 25-50% ammonium sulphate precipation was applied on to Octyl-Sepharose CL-4B column (30 ml) in the presence of 0.1 M $CaCl_2$ at room temperature. Elution was carried out with 0.1 mM EDTA in 5mM PIPES, pH 7.0. Fractions 28-31 with highest PLD activity were collected. The protein content in fractions was determined by absorbance at 280 nm and the PLD activity was monitored with standard assay described in section 4.9.1.1.

Initial attempts of performing calcium-mediated hydrophobic interaction chromatography on Octyl-Sepharose CL-4B with the crude extract of mustard seeds according to the established protocol for cabbage PLD (Lambrecht and Ulbrich-Hofmann, 1992) was unsuccessful, because PLD activity did not bind to the matrix rather it was present in the unbound fractions. Obviously, there were some unknown components present in the crude extract of mustard seeds that interfere in the binding of PLD activity on to the hydrophobic matrix. Therefore, a two step ammonium sulphate precipitation (i.e. 0-25% followed by 25-50%) of the crude extract was carried out followed by extensive dialysis as described in section 4.1.3. This step proved useful in getting rid of interfering components and subsequently in carrying out the next step of purification which involved the interaction of PLD with the hydrophobic matrix of Octyl-Sepharose CL-4B in the presence of calcium ions. Interestingly, in case of mustard PLD, calcium-mediated hydrophobic interaction chromatography on Octyl-Sepharose CL-4B was performed at room temperature where as previous attempts to carry out the process at 4-5^{o}C as used in the reference protocols (Lambrecht and Ulbrich-Hofmann, 1992; Schaeffner *et al.,* 2005; Oblozinsky *et al.,* 2003) were unsuccessful. When the hydrophobic interaction

chromatography on Octyl-Sepharose CL-4B was carried out under cold conditions, most of the PLD activity was observed in flow through. However, at room temperature more than 98% of the PLD activity bound to the matrix in the presence of calcium ions (0.1 M). Few other contaminating proteins which also bind to the matrix under these conditions and subsequently got eluted along with PLD after the calcium ions has been chelated using EDTA (in the elution buffer) were successfully removed after the subsequent step of anion exchange chromatography as can be viewed from fig 5.3.

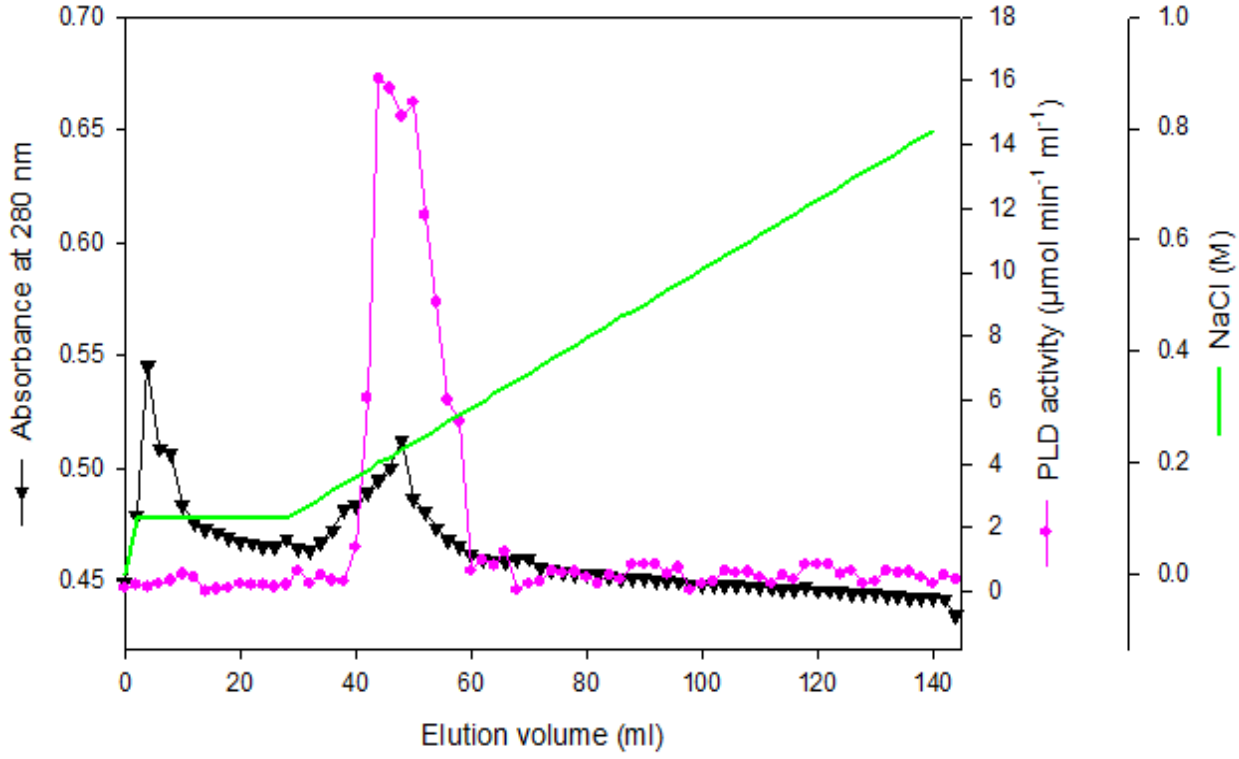

Fig. 5.2. Elution profile of mustard PLD on Source 15 Q column. 80 ml of pooled and active fractions obtained after calcium-mediated hydrophobic interaction chromatography was applied on to Source 15Q column (3.6 ml). Binding and washing of unbound proteins was carried out with 10 mM PIPES, pH 7.0 buffer and the elution was performed with a linear gradient of 0.1-0.75 M NaCl in 10 mM PIPES, pH 7.0. The PLD activity was monitored by standard assay as described in section 4.9.1.1.

The specific activity and the recovery yield after each step of purification were summarized in Table 5.1. The SDS-PAGE profile after the different purification steps shows purification of PLD to apparent homogeneity after anion exchange chromatography (fig 5.3). From 100g of dry seeds 0.208 mg of pure PLD was obtained with the specific activity of 254 µmol min^{-1} mg^{-1} as determined by standard assay (section 4.9.1.1).

Table 5.1. **Purification of PLD from mustard seeds.** PLD activity was measured by the standard activity assay (described in section 4.9.1.1). The protein content in the crude extract of mustard seeds and in samples after ammonium sulphate precipitation was determined by the Bradford assay (section 4.2) while in samples after calcium mediated hydrophobic interaction chromatography and anion exchange chromatography was quantified by Micro BCA protein assay (section 4.2).

Purification step	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude extract	3,390	366	0.108	1	100
Ammonium sulphate precipitation (25-50%)	1,476	230	0.156	1.44	63
Hydrophobic interaction chromatography on Octyl-Sepharose CL-4B	1.6	166	104	963	45
Anion exchange chromatography on Source 15Q	0.208	53	254	2351	14

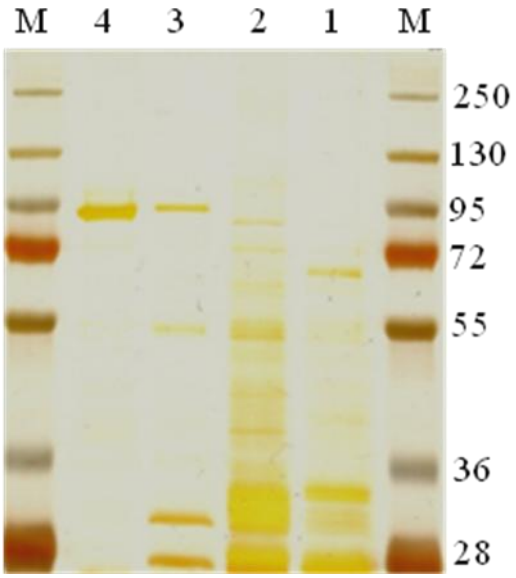

Fig 5.3. SDS-PAGE profile of PLD purification from mustard seeds. SDS-PAGE was carried out according to section 4.3. Lane M, pre-stained molecular markers with their indicated molecular masses in kDa; lane 1, crude extract of mustard seeds; lane 2, dialyzed sample after ammonium sulphate precipitation; lane 3, pooled active fractions after chromatography on Octyl-Sepharose CL-4B; lane 4, pooled active fractions after chromatography on Source 15Q. The gel was stained with silver.

5.2. Molecular characterization of the PLD

5.2.1. Molecular mass and activity staining

The molecular mass of the purified mustard PLD was determined by SDS-PAGE using 10% gels under reducing conditions as described in section 4.3. The mustard PLD displayed a single band on SDS-PAGE gel (fig 5.4A) and the apparent molecular mass of PLD as calculated from the standard proteins (described in section 4.3) was 91.9 ± 1.9 kDa (fig 5.4B).

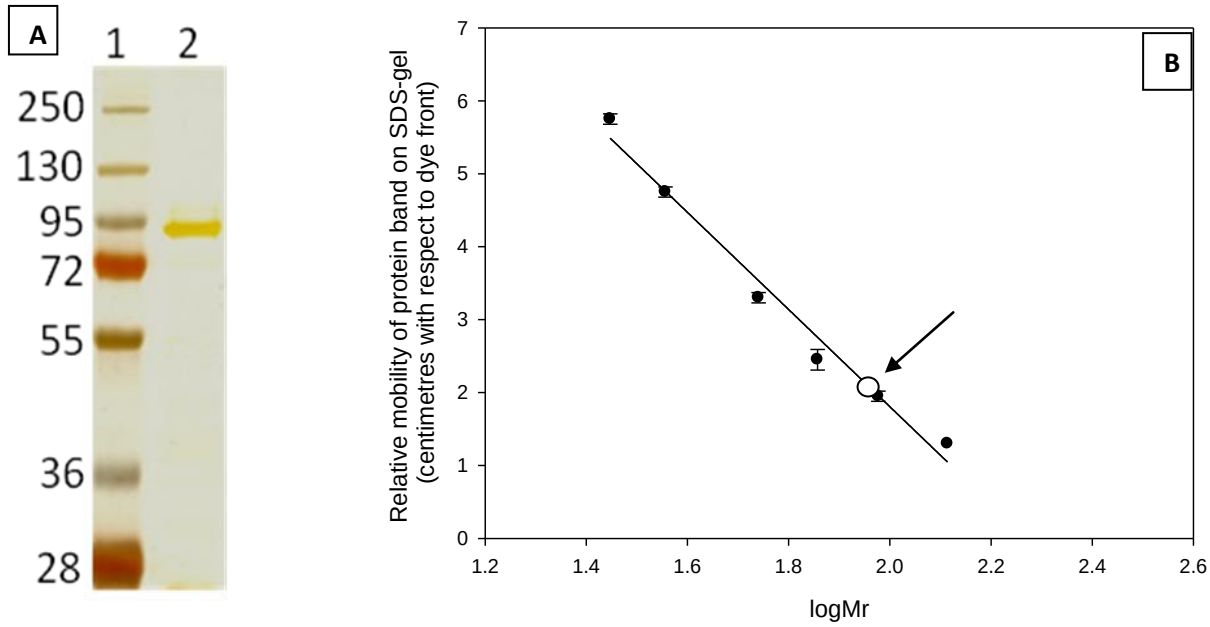

Fig. 5.4. Molecular mass determination of mustard PLD. (A) SDS-PAGE (10%) of mustard PLD. Lane 1, protein markers with their indicated molecular masses; lane 2, purified mustard PLD. **(B)** The semi-logarithmic plot of relative mobility versus molecular mass. Solid and open circles represent values of standard molecular markers and mustard PLD (indicated with arrow) respectively.

NATIVE-PAGE of mustard PLD was performed according to section 4.5. Fig 5.5A demonstrates the NATIVE-PAGE of the PLD displaying single band in each lane. In another experiment, the NATIVE-PAGE of PLD was performed with increasing amount of enzyme and subsequently the protein bands from the gel were transferred onto the nitrocellulose membrane followed by activity staining of membrane as described in section 4.6. The PLD retained its activity upon transfer to the membrane as demonstrated by the pink colour spots which appeared on the membrane blot as a result of PC hydrolysis by mustard PLD (fig 5.5B). The appearance of a single pink coloured spot in each lane on the membrane blot which corresponds to the single band on the NATIVE-gel (fig 5.5A) together with the single band of mustard PLD obtained in SDS-PAGE (fig 5.4A) clearly indicates that the purified preparation is homogenous.

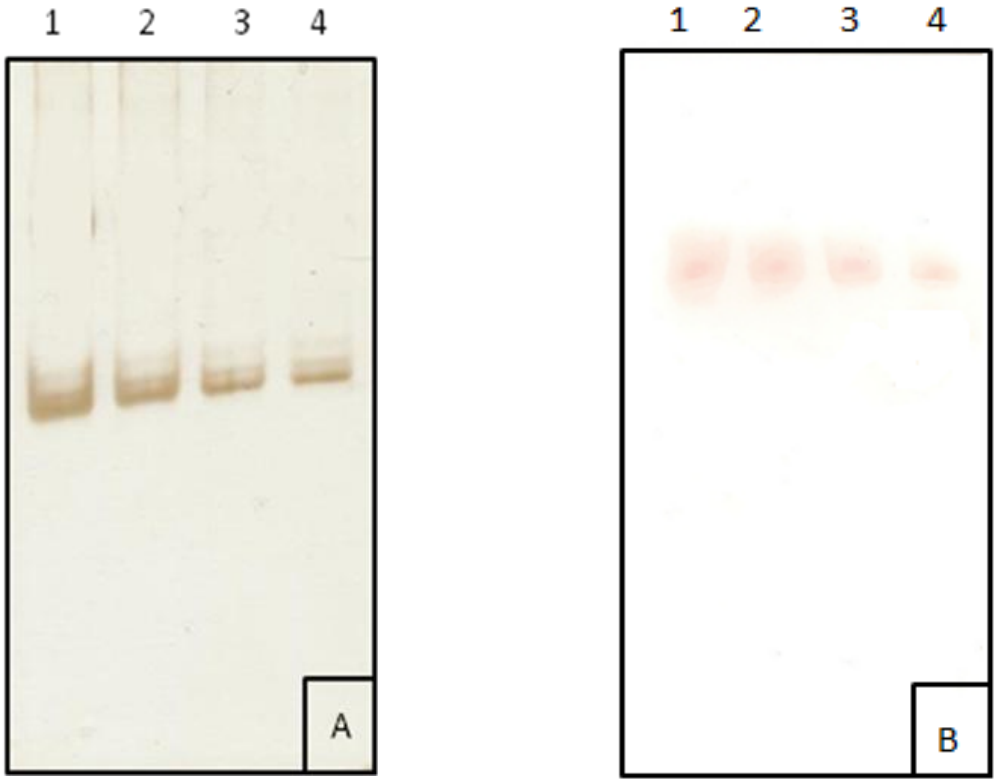

Fig. 5.5. NATIVE-PAGE (A) and activity stained blot membrane (B). (A) The electrophoresis was carried out under non denaturing conditions at 6°C follwed by silver staining as described in section 4.5. Lanes 1-4; 0.10, 0.21, 0.42 and 0.52 µg of the purified PLD **(B)** Activity stained nitrocellulose membrane showing pink spots as a result of PLD activity on PC substrate (described in section 4.6). Lanes 1-4; 0.10, 0.21, 0.42 and 0.52 µg of the purified PLD.

5.2.2. Isoelectric point (pI) and immunogenicity

The isoelectric point (pI) of mustard PLD was determined as described in section 4.7. As can be seen in fig 5.6A, the mustard PLD appeared as a single band (lane 2) in the gel along with the isoelectric focussing (IEF) protein markers (pH 3-10) (lane 1), which indicates that there are no isoforms of mustard PLD present in the purified preparation. The pI value of mustard PLD as estimated from fig 5.6A is in the range of 4.3-4.4.

To examine whether mustard PLD has any structural relationship with other plant PLDs, immunoblotting of the enzyme was carried out using the polyclonal antibodies generated against poppy PLDα1 (explained in section 4.4). As shown in fig 5.6B, the antibodies raised against purified poppy PLD cross reacted with mustard PLD. Also, the migration rate of both mustard and poppy PLD was similar and corresponds to the molecular mass of around 92 kDa. The interaction of anti-poppy PLDα1 with mustard PLD suggests that the mustard PLD has structural regions which are similar to poppy PLDα1.

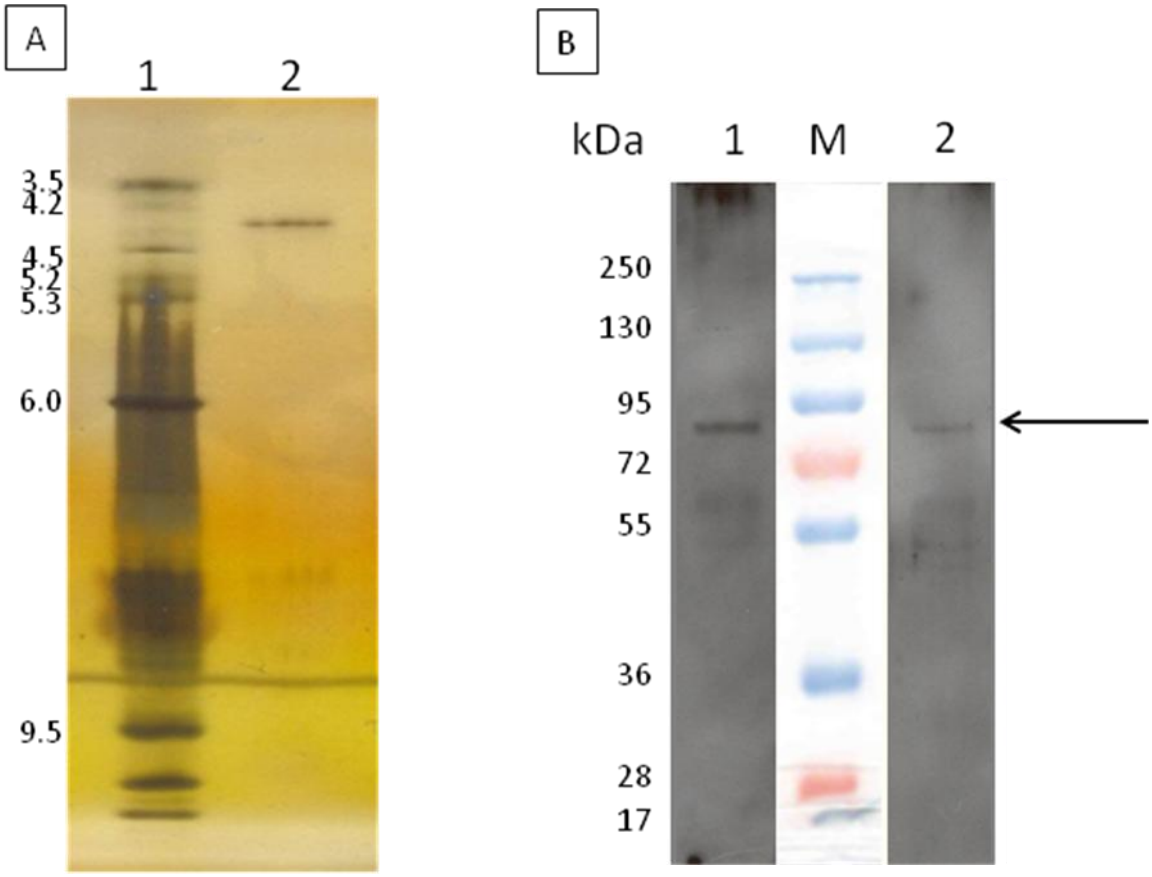

Fig. 5.6. Isoelectric focussing and immunoblott of mustard PLD. **(A)** Isoelectic focussing of mustard PLD (lane 2) and standard proteins with their indicated isoelerctric points (lane 1). The experiment was performed with Servalyt-Precotes 3-10 and the IEF markers from Serva (pH 3-10) as described in section 4.7. **(B)** Immunoblotting was performed with mustard PLD and poppy PLDα1 using antibodies against poppy PLDα1 as described in section 4.4. lane 1, PLD1 from poppy; lane M, prestained molecular markers; lane 3, mustard PLD (shown with arrow).

5.2.3. Partial sequence alignment

To obtain information on the similarity of the mustard PLD to the well characterized PLDs from other plant sources, the enzyme was subjected to mass spectrometric analysis as described in section 4.8. The amino acid sequences from mustard PLD obtained by mass spectrometry were compared to amino acid sequences of known PLDs by BLAST using NCBI website (http:/www.ncbi.nlm.nih.gov/BLAST/). The alignment was performed using MULTIALIN software (Corpet, 1988). Fig 5.7 presents the alignment of the amino acid sequences of peptides obtained from mustard PLD to that of the sequences of two well known PLDs from plants: PLDα2 from cabbage (*Brassica oleracea* var. capitata) (GenBank ID: AAC78486.1) and PLDα1 from poppy (*Papaver somniferum*) (GenBank ID: AAL48261.2). As observed from the fig 5.7, the peptides from mustard PLD demonstrate 100% and 85% sequence identity respectively, to the amino acid sequences of the cabbage PLDα2 and poppy

PLDα1. Moreover, the two longest peptide fragments from mustard PLD with amino acid sequences *ddnpigatligrayvpvdevingeevek* and *vlllvwddrtsvdvlkkdglmathdedtenyfngsevhcvlcpr* shared very high sequence identity to PLDs from other plant sources specifically to α-type PLDs (table 5.2). Table 5.2 demonstrates the identity of both of the above sequences to the α-type PLDs from different plant sources were in the range of 100-80% but show either relatively low or no sequence identity to β-, δ-, γ- and ζ- types of PLDs from *A. thaliana* (For reference to classification of plant PLDs see section 2.2.1.2).

```
          1                                                   50
PLDcab    MAQHLLHGTL HATIYEVDAL HTGGLRSAG. ..FLGKIISN VEETIGFGKG
PLDpop    MAQISLHGTL HVTIFEANSI SHPDRKTGGA PKFFRKLVEN IEETVGFGKG
PLDmus    .......... .......... .......... .......... .........G

          51                                                  100
PLDcab    ETQLYATIDL QKARVGRTRK ITDEPKNPKW YESFHIYCAH MASDIIFTVK
PLDpop    ASMLYASVDL DKARVGRTRI IKDEPVNPKW YESFHIYCAH MAANVIFTVK
PLDmus    ETQLYATIDL QK........ .......... .......... ..........

          101                                                 150
PLDcab    DDNPIGATLI GRAYVPVDEV INGEEVEKWV EILDDDRNPI HGESKIHVKL
PLDpop    DDNPIGATLI GRAYVPIDKV LSGEEVDEWV EVVDQERNPV QGGCKIHVKL
PLDmus    DDNPIGATLI GRAYVPVDEV INGEEVEK.. .......... ..........

          151                                                 200
PLDcab    QYFAVEADRN WNMGVKSAKF PGVPYTFFSQ RQGCKVSLYQ GAHVPDNFVP
PLDpop    QYFDVGQDKN WARGIRSAKF PGVPYTFFTQ RTGCKVSLYQ DAHVPDNFIP
PLDmus    .......... ........KF PGVPYTFFSQ R....VSLYQ GAHVPDNFVP

          201                                                 250
PLDcab    KIPLAGGKNY EPHRCWEDIF DAITNAKHLI YITGWSVYTE ITLVRDSRRP
PLDpop    KIPLAGGKIY EPARCWEDIF DAISNAKHMI YITGWSVYTE VVLIRDSRRQ
PLDmus    K......... ....CWEDIF DAITNAK... .......... ..........

          251                                                 300
PLDcab    KPGGDMTLGE LLKKKATEGV RVLLLVWDDR TSVDVLKKDG LMATHDEDTE
PLDpop    KPGGDITLGE LLKKKADEGV RVLMLVWDDR TSVGLLKKDG LMATHDEDTF
PLDmus    .......... .......... .VLLLVWDDR TSVDVLKKDG LMATHDEDTE

          301                                                 350
PLDcab    NYFNGSEVHC VLCPRNPDDG GSIVQNLQVS AMFTHHQKIV VVDSEVPSQG
PLDpop    NYFQGTQVNC VLCPRNPDDG GSFIQDLQIS TMFTHHQKIV VTDSAMPSEG
PLDmus    NYFNGSEVHC VLCPR..... .......... .......... ..........

          351                                                 400
PLDcab    GGSEMRRIMS FVGGIDLCDG RYDTPFHSLF RTLDTVHHDD FHQPNFTGAS
PLDpop    ..SQQRRIVS FVGGIDLCDG RYDTQFHSLF RTLDTAHHDD FHQPNFTGGA
PLDmus    .......IMS FVGGIDLCDG RYDTPFHSLF R......... ..........
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         401                                                450
PLDcab   ITKGGPREPW QDIHSRLEGP IAWDVLYNFE QRWSKQGGKD ILVKLRELSD
PLDpop   ITKGGPREPW HDIHSCLEGP IAWDVLFNFE QRWRKQGGKD ILVNLRELSE
PLDmus   .......EPW QDIHSRLEGP IAWDVLYNFE QR..................

         451                                                500
PLDcab   IIITPSPVMF QEDHDVWNVQ LFRSIDGGAA AGFPDSPEVA AEAGLVSGKD
PLDpop   IIIPPSPVTF SEDPESWNVQ LFRSIDGGAA FGFPDSPEDA AKAGLVSGKD
PLDmus   .......... .......... .......... .......... ..........

         501                                                550
PLDcab   NVIDRSIQDA YIHAIRRAKD FIYIENQYFL GSSFAWAADG ITPEDINALH
PLDpop   NIIDRSIQDA YINAIRRAKD FIYIENQYFL GSSYGWKADG IKPEEINALH
PLDmus   .....SIQDA YIHAIR.......... ........................

         551                                                600
PLDcab   LIPKELSLKI VDKIEKGEKF RVYVVVPMWP EGIPESASVQ AILDWQRRTL
PLDpop   LIPKELSLKI VSKIEAGERF TVYVVVPMWP EGIPESASVQ AILDWQRRTW
PLDmus   .........I VDKIEK. .......... .......... .......... TL

         601                                                650
PLDcab   EMMYKDVTQA LRAQGLEEDP RNYLTFFCLG NREVKKEGEY EPAERPDPDT
PLDpop   DMMYADITQA LKAKGLVADP RDYLTFFCLG NREVKKEGEY VPSETPDPDT
PLDmus   EMMYKDVTQA LRAQGLEEDP R......... .....KEGEY EPAERPDPDT

         651                                                700
PLDcab   DYMRAQEARR FMIYVHSKMM IVDDEYIIVG SANINQRSMD GARDSEIAMG
PLDpop   DYSRAQAARR FMIYVHTKMM IVDDEYIIIG SANINERSMN GARDSEIAMG
PLDmus   DYMR.......... ...... .......... .......... ..........

         701                                                750
PLDcab   GYQPHHLSHR QPARGQVHGF RMSLWYEHLG MLDETFLDPS SLECIEKVNR
PLDpop   GYQPHHLAHR EPATGQIHGF RMALWYEHLG MLDEVFLHPN SEECVQKVNR
PLDmus   .......... ....GQVHGF R......... .......... ..........

         751                                                800
PLDcab   IADKYWDFYS SESLEHDLPG HLLRYPISVD NEGNITELPG FEFFPDSKAR
PLDpop   IADKYWDLYS SDSLERDLPG HLLRYPIGIT ENGDVTALPG HEFFPDTKAR
PLDmus   .......... .......... .......... .......... ..........

         801          815
PLDcab   ILGNKVDYLP PILTT
PLDpop   VLGGKSDYLP PILTT
PLDmus   .......... .....
```

Fig. 5.7. Partial sequence alignment. Partial amino acid sequences of trypsin-digested PLD from mustard seeds (PLDmus) obtained after mass spectrometric analysis (MS / MS data search) compared with sequences of PLDα2 from cabbage (PLDcab) and PLDα1 from poppy (PLDpop) using BLAST. Amino acids (2-150) represents C2 domain, 204-393 and 496-704 represents active site (numbering of amino acid residue is related to PLDcab). The alignment was performed using MULTIALIN software.

Table 5.2. The sequence identity of the two largest amino acid fragments of mustard PLD with the PLDs from other plant sources. n.d means not determined.

PLD sources	Sequence identity (%)	
	DDNPIGATLIGRAYVPVDEVINGEEVEK	VLLLVWDDRTSVDVLKKDGLMATHDEDTENYFNGSEVHCVLCPR
PLDα2 (*Brassica oleracea*)	100	100
PLDα1 (*Arabidopsis lyrata*)	93	94
PLDα1 (*Arabidopsis thaliana*)	89	94
PLDα1 (*Brassica oleracea*)	79	94
PLDα2 (*Arabidopsis thaliana)*	n.d	88
PLDs (*Oryza sativa)*	70	84
PLD1 (*Papaver somniferum*)	82	88
PLDα1 (*Vitis vinifera*)	75	82
PLDα (*Populus trichocarpa*)	n.d	88
PLDα2 (*Arachis hypogoea*)	81	84
PLDα1 (*Papaver somniferum*)	82	88
PLDα2 (*Papaver somniferum*)	81	88
PLDα (*Citrus sinensis*)	82	78
PLDα (*Medicago truncatula*)	81	78
PLDα1 (*Nicotiana tabacum*)	79	78
PLDα1 (*Glycine max*)	81	78
PLDζ (*Arabidopsis thaliana*)	63	69
PLD β (*Arabidopsis thaliana*)	0	62
PLD δ (*Arabidopsis thaliana*)	0	59
PLD γ (*Arabidopsis thaliana*)	0	53
PLD β1 (*Arabidopsis thaliana*)	0	62
PLDβ2 (Solanum lycopersicum)	n.d	62
PLDδ (*Glycine max*)	n.d	59

5.3. Characterization of the PLD in mixed micellar system

5.3.1. Kinetic parameters of the PLD

As explained in section 2.1 the substrates of PLD form complex structure in water, therefore making the investigation of the kinetic behaviour of such enzymes are much more difficult than that of the hydrolytic enzymes whose substrate are water soluble.

Thus in order to study the kinetics of mustard PLD, the natural substrate PC in a mixed micellar system was employed. The substrate solution containing PC and detergents (SDS and Triton X-100) in the molar ratio 1: 0.5: 1.6 was prepared as described in section 4.9.1.1 and the effect of substrate concentration on the activity of PLD was investigated at pH 5.5 and 37°C as illustrated in section 4.9.2. The PLD activity increases hyperbolically with the increase in substrate concentration and could be fitted to Michaelis-Menten equation (fig 5.8). The inset of fig 5.8 showed the corresponding Lineweaver Burk plot. The values of V_{max} and K_m as calculated from the graph were 356 ± 15 μmolmin^{-1}mg^{-1} and 1.84 ± 0.17 mM, respectively.

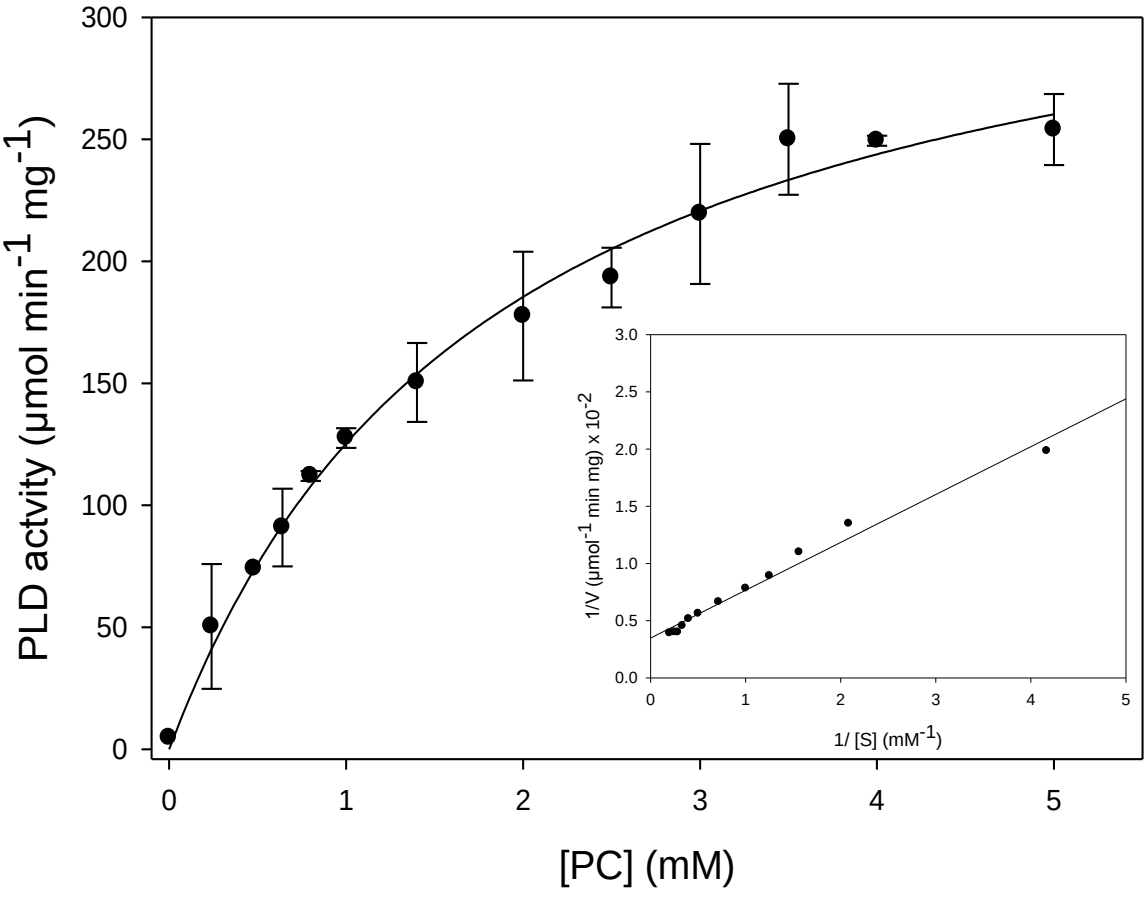
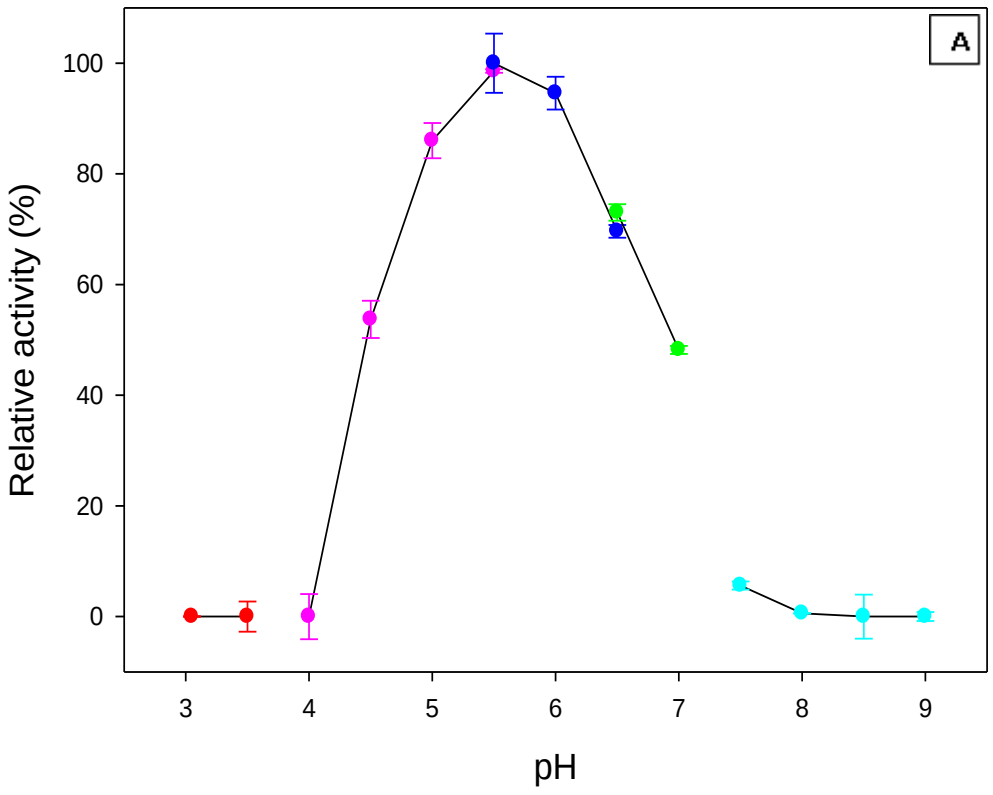


Fig. 5.8. PLD activity as a function of substrate concentration. *v*/[S] characterstic of the PLD for PC was taken in a mixed micellar system using PC, SDS and Triton X-100 in a molar ratio of 1:0.5:1.6. The inset showed the Lineweaver-Burk plot of the data. Activity of the PLD was measured at varying concentrations of substrate (0-5 mM) under the standard assay conditions of 70 mM $CaCl_2$, pH 5.5 and 37°C as described in section 4.9.2.

5.3.2. Effect of pH on the activity and stability of the PLD

The effect of pH on the hydrolytic activity of mustard PLD was studied in the range pH 3.0-9.0 using five buffering systems (explained in section 4.9.3). As demonstrated in fig 5.9A, the purified PLD showed optimum activity in the relatively wide pH range of 5.5-6.0 and the activity decreased strongly above pH 7.0 or below pH 4.5.

The pH range of optimal stability of the mustard PLD was investigated by incubating the purified enzyme in different buffers (pH 4.5-10.0) for 1.5 hours at room temperature and then analyzing the activity at pH 5.5 under standard assay conditions as described in section 4.9.3. The activity of PLD at different pH values relative to its activity without pre-incubation was plotted as a function of pH. As depicted in fig 5.9B, the mustard PLD was moderately stable at a relatively broad pH range (pH 5.0-9.0) and the enzyme showed remarkable increase in stability as the pH increases from 7.0 (60 % relative activity) to 9.0 (90 % relative activity), showing maximum stability at pH 8.5 (100 % relative activity) and then there was a tremendous decrease in stability at pH 10.0 (with the loss of almost 80% activity relative to non incubated enzyme).



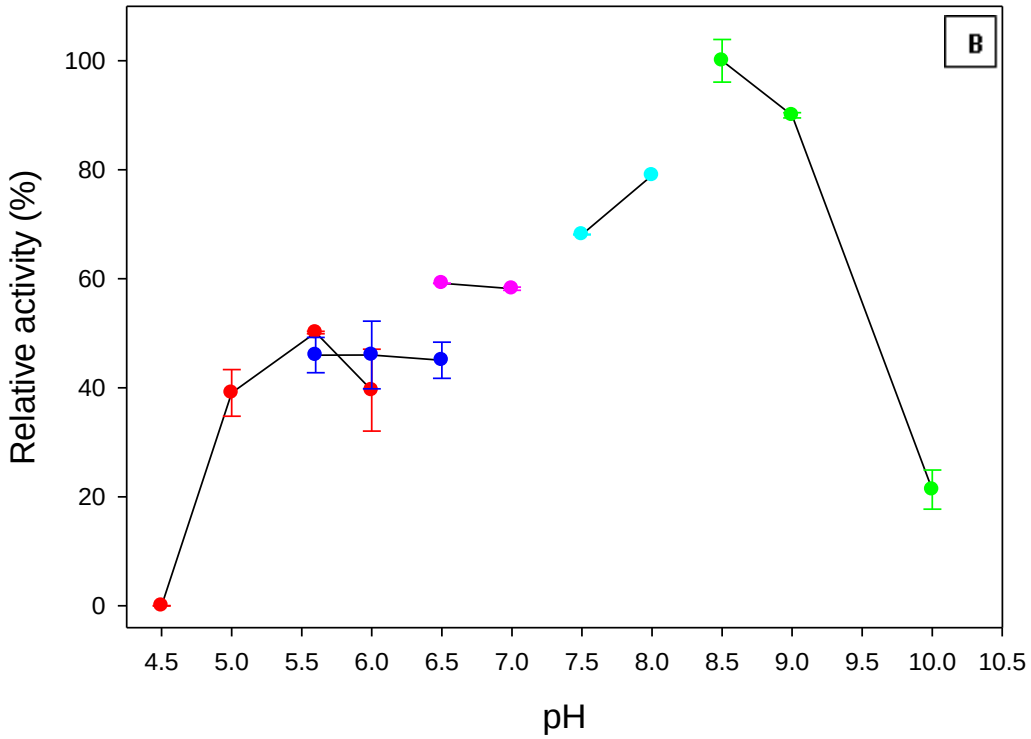


Fig. 5.9. Effect of pH on the PLD activity and stability. (A) PLD activity as a function of pH. Enzyme activity was measured by standard assay but the buffers used were sodium-formate (pH 3.0-3.5), sodium acetate (pH 4.0-5.5), MES (pH 5.5-6.5), HEPES (pH 6.5-7.0), Tris-HCl (pH 7.5-9.0) each containing 70 mM calcium chloride (described in section 4.9.3). **(B)** PLD stability as a function of pH. The enzyme was incubated in each buffer solutions for 1.5 h and then the activity was analyzed under standard assay conditions at pH 5.5 (described in section 4.9.3). The buffers (each 50 mM) used were sodium-acetate (pH 4.0-5.5), MES (pH 5.5-6.5), PIPES (pH 6.5-7.0), HEPES (pH 7.5-8.0) and glycine (pH 8.5-9.0).

5.3.3. Effect of temperature on the activity and stability of the PLD

The effect of temperature on the activity and stability of mustard PLD was investigated as described in section 4.9.4. In fig 5.10A, the PLD activity relative to the activity of PLD at 37°C was plotted as a function of temperature. The graph shows a relatively steep temperature optimum around 45°C.

PLD undergoes irreversible thermal unfolding so the activity presented at higher temperatures (fig 5.10A) actually correlates to the conversion rate of substrate by the still active fraction of PLD. Fig 5.10B shows the Arrhenius plot derived from the data of the increasing branch of the fig 5.10A. The activation energy of PLD as calculated from the slope of the straight line (explained in section 4.9.4) was 8.8 kcal mol^{-1}.

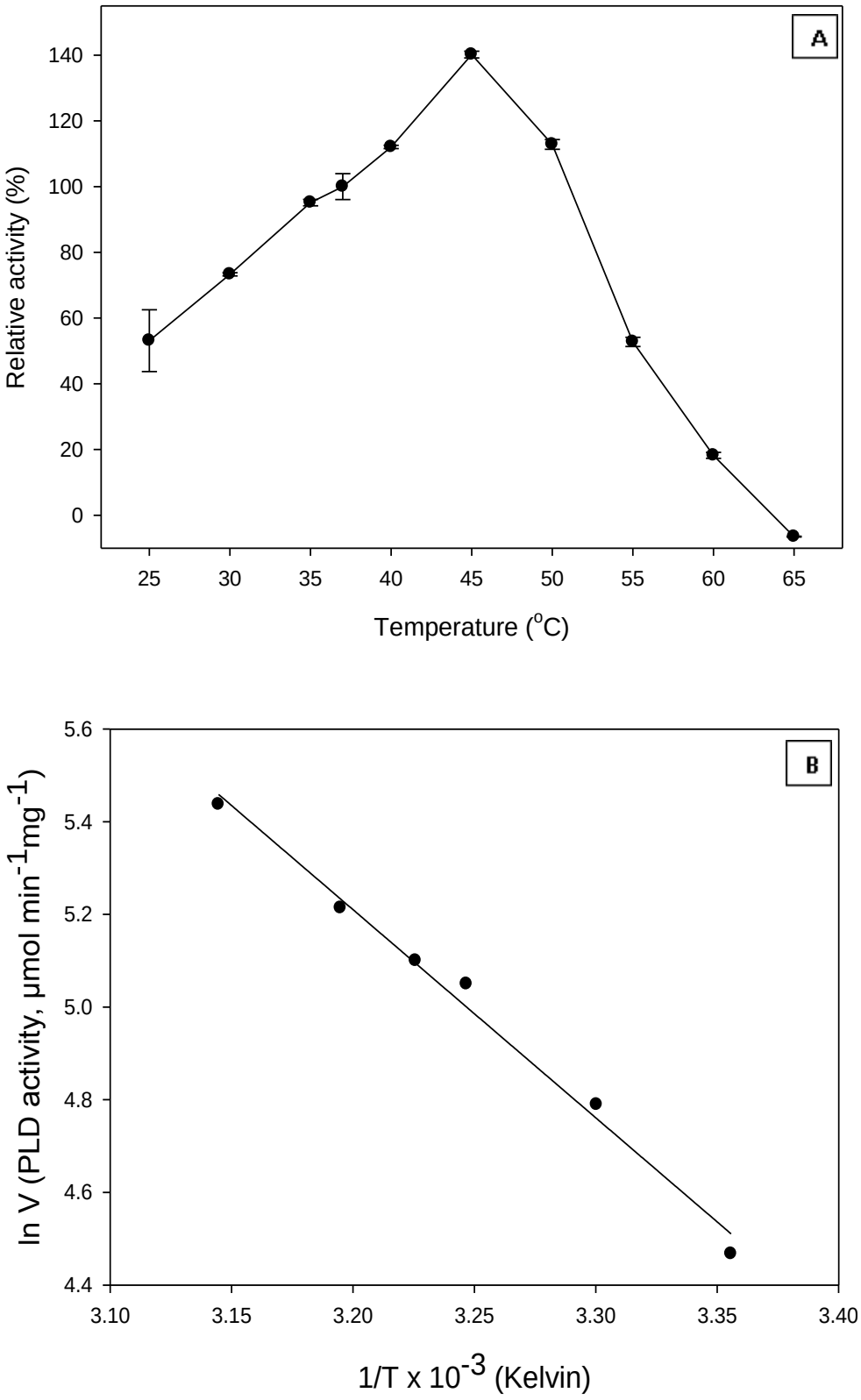


Fig. 5.10. The influence of temperature on the activity of PLD. (A) PLD activity (relative to its activity at 37°C) as a function of temperature. Enzyme was assayed at different temperatures (25°C-70°C) as described in section 4.9.4., and the activity was plotted as a function of temperature. **(B)** Arrhenius plot of the data from (A). The experiments were conducted in duplicates and the variations were less than 5%.

The thermal stability of the enzyme was determined by assaying PLD samples in 0.05 M PIPES buffer at pH 7.0 after the exposure to various temperature levels for 8 min as illustrated in section 4.9.4. As clear from fig 5.11, the enzyme was stable for a relatively narrow range of temperature from 25 up to 40°C. The enzyme retained almost 60% activity after incubation at 50°C followed by a remarkable loss of more than 90% activity after incubation at 55°C.

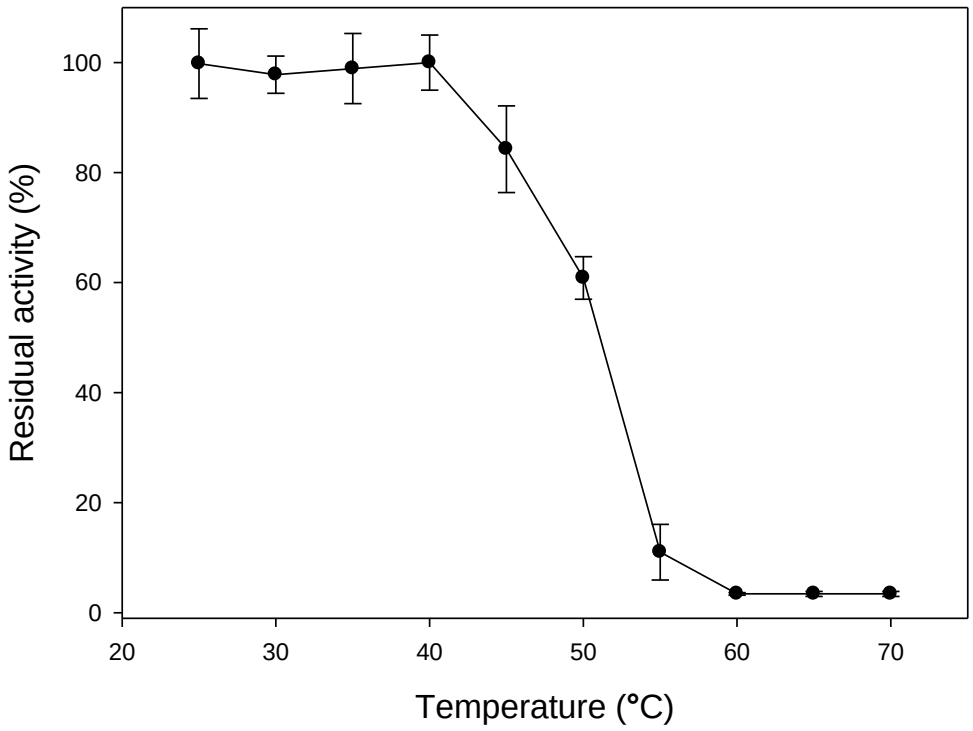


Fig. 5.11. Influence of temperature on the stability of the PLD. The enzyme was pre-incubated for 8 min at different temperatutes (25-70°C) followed by immediate chilling on ice. The activity was measured by standard assay at 37°C and pH 5.5 as described in section 4.9.4. The experiments were conducted in duplicates.

5.3.4. Effect of metal ions on the activity and stability of the PLD

The effect of metal ions on the activity of PLD was investigated with the standard assay as described in section 4.9.6. Table 5.3 displays PLD activity in the presence of various metal ions related to its activity in the presence of calcium ions, which possess the highest activity effect on PLD. No activity was detected in the absence of metal ions which implies that the presence of metal ions is mandatory for the activity of PLD. Mustard PLD showed low but significant activities in the presence of magnesium, zinc and manganese ions while no activity was detected in the presence of potassium, nickel or lithium ions.

Table. 5.3 The influence of metal ions on the activity of the PLD. PLD activity was determined by standard assay at 37°C and pH 5.5 except that the reaction buffer contained 100 mM of different metal chlorides (described in section 4.9.6). The experiments were conducted in duplicates. b.d stands for the values that are below the detection limit of the activity assay system used.

Metal ions (chloride salts) (100mM)	**Relative activity (%)**
----	b.d.
Calcium	100 ± 5
Zinc	3.0 ± 0.6
Manganese	4.8 ± 1.8
Magnesium	3.0 ± 2.3
Potassium	b.d.
Lithium	b.d.
Nickel	b.d.

Calcium ions, though critical for the activity of PLD were also shown to destabilize protein conformation (Younus *et al.*, 2003; Stumpe *et al.*, 2007). Therefore, the effect of calcium ions on the stability of PLD was studied by incubating the enzyme in 40 mM $CaCl_2$ solution (pH 7.0) for different intervals of time at room temperature (described in section 4.9.5). The enzyme was also incubated with NaCl solution of the same ionic strength so as to know the influence of ionic strength on the stability of PLD. As shown in fig 5.12, the activity of PLD incubated with $CaCl_2$ and NaCl solution decreases continuously with increasing incubation time as compared to the non-incubated PLD (designated as control in the fig 5.12). As compared to 40% reduction of activity in control after 90 min, the enzyme looses about 60% of its activity in the presence of salts. Moreover, irrespective of the type of salts (NaCl or $CaCl_2$), if used at the same ionic strength, the loss of enzyme activity is same at all intervals of time. This showed that the instability of PLD was caused by the high ionic strength rather than by the specific ionic effects.

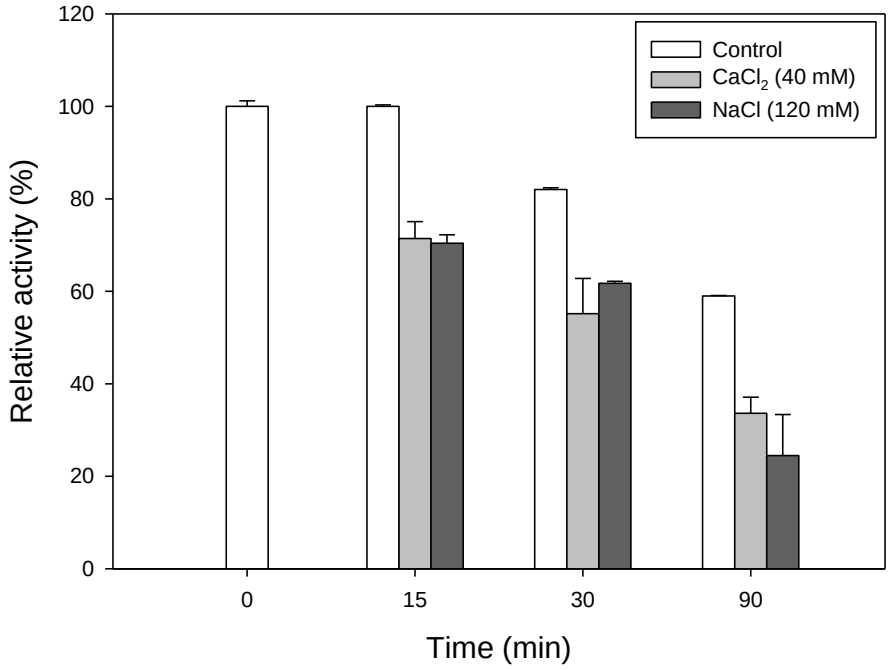


Fig. 5.12. The influence of metal ion salts on the stability of PLD. The enzyme was incubated in 10 mM PIPES pH 7.0 containing 40 mM $CaCl_2$ or 120 mM NaCl for different time intervals (15-90 min) at room temperature followed by measuring activity by standard assay at 37°C and pH 5.5 (described in section 4.9.5). The experiments were conducted in duplicates.

5.3.5. Specification of the effect of Ca^{2+} ions on the activity of the PLD

The effect of calcium ions on the activity of PLD was investigated more extensively as described in section 4.9.6. Fig 5.13A shows the PLD activity as a function of calcium ion concentration. At concentrations of calcium ions less than 1 mM no significant activity could be detected. However, as the calcium ions concentration increases (beyond 1 mM) the activity of mustard PLD increases till it reached a maximum at around 60 mM. Further increase of calcium ions in the reaction medium (up to 200 mM) did not resulted in rise of the PLD activity. The activity data in fig 5.13A could be well fitted using a double hyperbolic function (SigmaPlot software, version 11.0) yielding two dissociation constants, K_{D1} = 0.146 mM and K_{D2} = 16.1 mM. A modified Scatchard plot of the data from fig 5.13A, with ΔR being the change in relative activity showed two linear parts, indicating that at least two separate calcium-binding events affect mustard PLD activity as shown in fig 5.13B (inset).

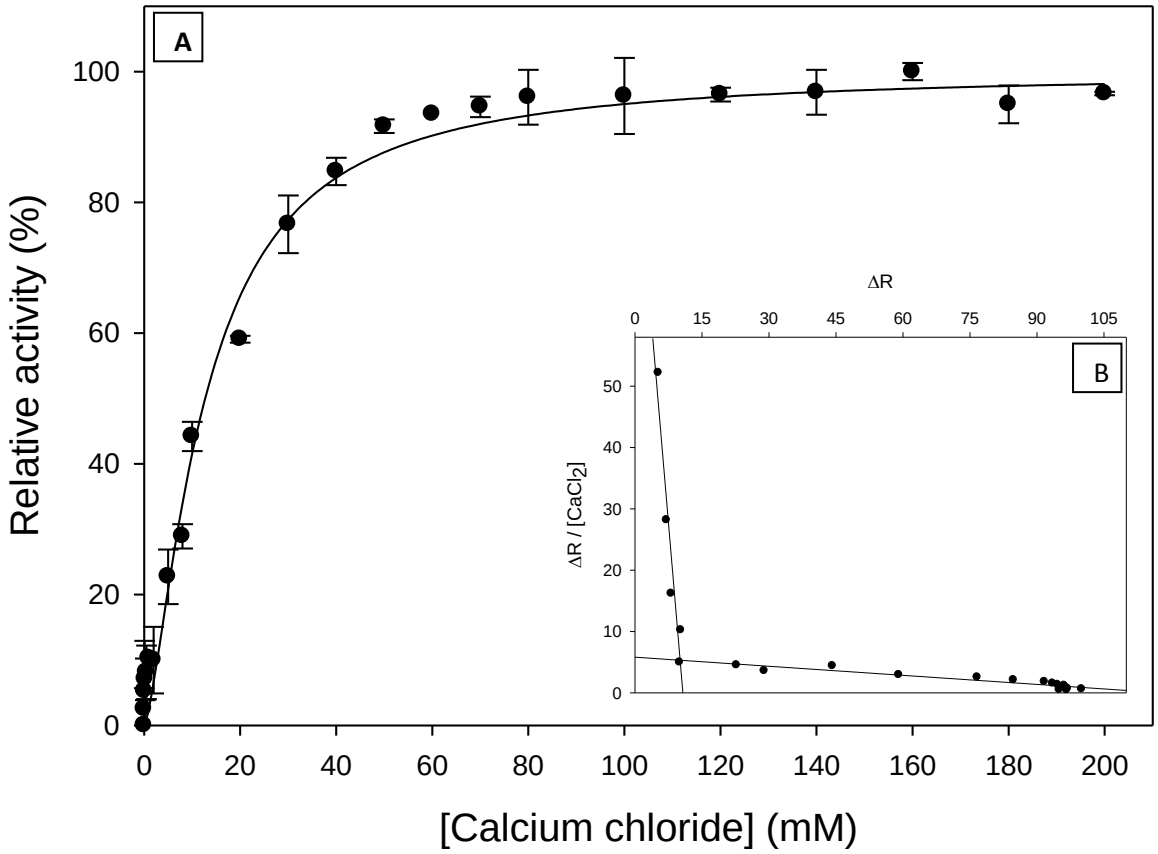


Fig. 5.13. Influence of Ca^{2+} ions concentration on the PLD activity. **(A)** Relative activity of PLD as a function of calcium chloride concentration (0-200 mM). The data were fitted using a double hyperbolic function (SigmaPlot, version 11.0). **(B)** Modified Scatchard plot of the data from (A). ΔR corresponds to change in relative activity. The PLD activity was measured with PC as substrate as described in section 4.9.6. The experiments were conducted in duplicates.

The activity of PLD as a function of pH was investigated at two different concentrations of calcium chloride (10 and 70 mM) as well as in its absence (as described in section 4.9.6). As demonstrated in fig 5.14, the pH optimum at all concentrations of Ca^{2+} ions remained in the same range of pH 5.5-6.0 and no significant shift in pH optimum was observed.

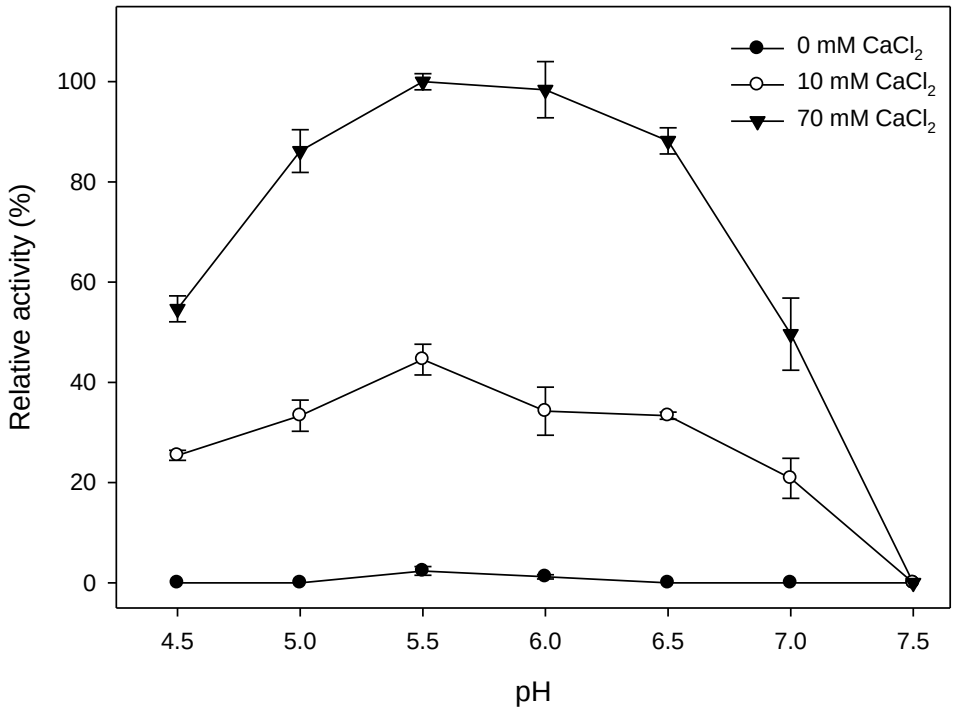


Fig. 5.14. PLD activity as a function of pH at different calcium ions concentration. The activity of PLD was measured with PC as substrate and the buffers used were 0.1 M sodium acetate (pH 4.5-5.0), 0.1 M MES (pH 5.5-6.0) and 0.1 M HEPES (pH 6.5-7.5) containing different concentrations of calcium chloride (0-70 mM) as described in section 4.9.6.

5.3.6. Effect of the substrate properties on the activity of the PLD

The effect of the chemical structure of substrate such as chain length, percent saturation of the fatty acyl groups, their positioning on the glycerol backbone as well as the composition of the alcoholic head group on PLD activity was investigated as described in section 4.9.7. The phospholipid substrates used in the study were prepared in the detergents at a molar ratio of PC:SDS:Triton X-100 of 1:0.5:1.6.

The effect of fatty acyl chain length of PC molecules on the activity of PLD is shown in fig 5.15A. The activity (related to the activity towards soya PC) was highest with the PC molecule containing an acyl chain of 8 carbon atoms (250%) followed by fatty acid chain length of 10 (150%). It decreased markedly when the fatty acid chains were longer than 10. Therefore, PC molecules with short chain fatty acids (C8-C10) are better substrates than PC with long chain of fatty acids (C12-C20).

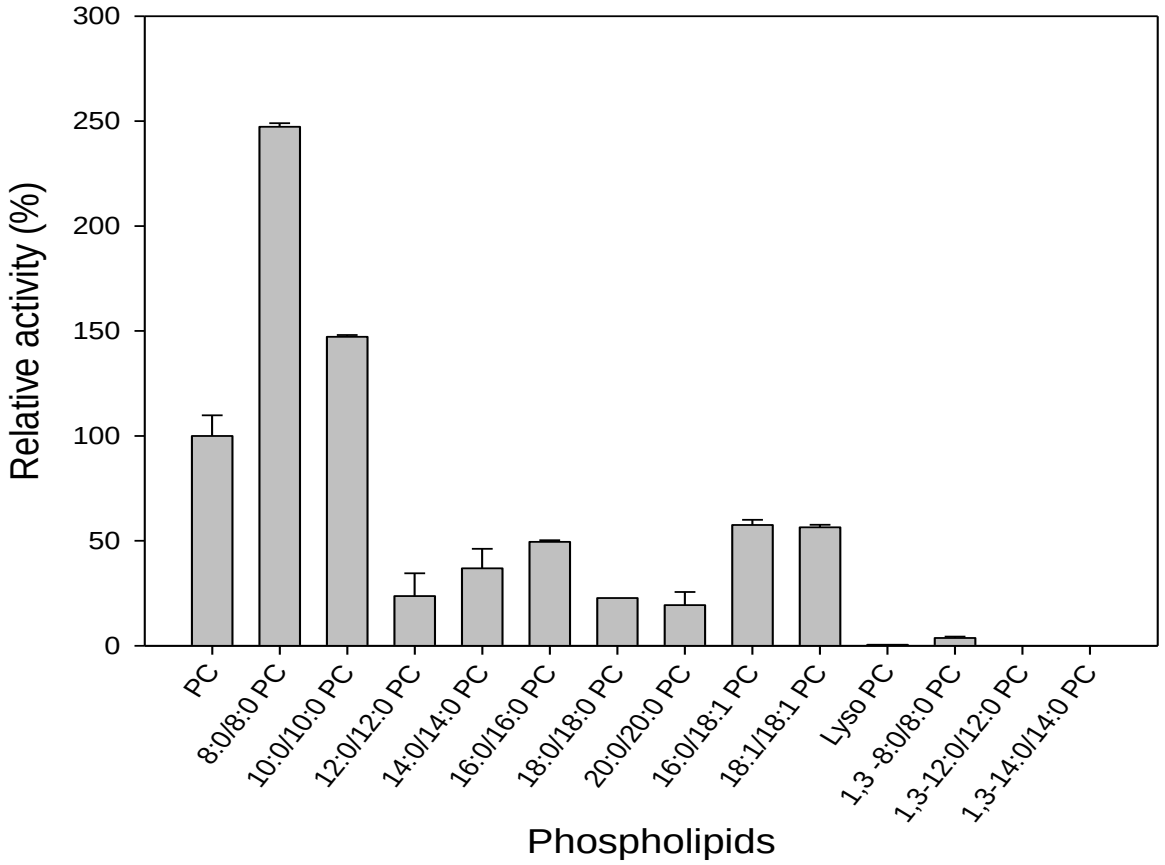


Fig 5.15. PLD activity as a function of chemical structure of PC substrate. The PLD activity was measured towards different PC substrates using assay conditions described in section 4.9.7. All the PC substrates used in the study were prepared in the detergents at a molar ratio of PC:SDS:Triton X-100 of 1:0.5:1.6. The final concentration of each PC substrate was 5mM.

Also from fig 5.15, it can be seen that the hydrolysis of PC with either one or both unsaturated fatty acids (16:0/18:1 and 18:1/18:1) in the *sn*-1 and *sn*-2 position, showed decreased but similar level of activity (~ 57%) as relative to soya PC. However, the hydrolytic rate of PC having both saturated (18:0/18:0) fatty acid chain was considerably lower (23%). Moreover, the level of hydrolysis of PC molecule having palmitic acid in both the *sn*-1 and *sn*-2 position (16:0/16:0 PC) increased only slightly when one of the palmitic acid was replaced by unsaturated oleic acid (16:0/18:1) i.e. from ~ 50% to 57% as relative to soya PC. These results indicated that the mustard PLD preferred PC molecules with unsaturated fatty acids over saturated ones and the preference depend upon the fatty acid chain length of PC molecules. In fig 5.15, it can also be observed that PLD possess almost no activity (~ 0.5%) with soya lyso PC. This showed that the two acyl chains are an absolute requirement for mustard PLD activity.

The activity of mustard PLD was also investigated with PC molecules having fatty acid chains in *sn*-1 and *sn*-3 positions of their glycerol backbone, designated as 1,3 PC in the present study. 1,3 PC used for this purpose consisted of three varying chain lengths i.e. 8:0/8:0, 12:0/12:0 PC and 14:0/14:0 PC, in their *sn*-1 and *sn*-3 positions respectively. As relative to the activity with soya PC, PLD possess negligible activity (only 3.7%) with 1,3 PC molecules having fatty acyl chain length of 8 carbon atoms but 1,3 PC molecules with the fatty acyl chain length of either 12 or 14 carbon atoms were not at all cleaved by the enzyme (fig 5.15).

Table 5.4 presents the activity of mustard PLD towards phospholipids of different head group alcohols (all from soya). It clearly indicates that PC was the most favourable substrate of mustard PLD followed by PE and PG.

Table 5.4 Substrate specificity of the PLD. Initial rates of hydrolysis of phosphatides (all from soya) with different alcoholic head groups. The activity of PLD with other substrates was presented as relative to its activity with PC. The activity was measured using the assay conditions described in section 4.9.7. All the phospholipid substrates used in the study were prepared in the detergents at a molar ratio of PC:SDS:Triton X-100 of 1:0.5:1.6. Data are the mean ± SD obtained from duplicate experiments.

Phospholipids	**Hydrolytic activity (µmol min^{-1} mg^{-1})**	**Relative activity (%)**
phosphatidylcholine	253.8 ± 13.4	100.0 ± 5.3
phosphatidylethanolamine	117.8 ± 9.8	46.4 ± 3.9
phosphatidylglycerol	43.6 ± 6.8	17.2 ± 2.7

5.3.7. Effect of detergents on the activity of the PLD

The effect of detergents on mustard PLD activity was studied with PC as substrate in the standard assay (section 4.9.1.1). Substrate solutions having different molar ratios of PC to detergent were prepared by mixing and co-sonicating different amounts of detergents with the fixed amount of PC (5 mM) as explained in section 4.9.8. However, with SDS and/or sodium deoxycholate, 40 mM Triton X-100 was mixed along with these detergents so as to avoid the precipitation or turbidity that appears during the reaction because of binding of Ca^{2+} ions to

the anionic amphiphiles. With all the PC-detergent ratios the concentration of PC was near the saturating range for PLD activity.

No PLD activity could be detected in the absence of detergent. At all molar ratio of PC to detergent studied, the maximum stimulation of enzyme activity was observed in the presence of SDS followed by sodium deoxycholate, CTAB and Triton X-100 (table 5.5). Little or no activity was detected in the presence of Brij-58 and Tween-20. Moreover, it can be observed from table 5.5 that as the molar ratio of PC to detergent increases, the enzyme activity increases, reaches a maximum and then declines again. The optimum PC/detergent ratio depends on the type of detergent used and ranges from 1:1.0 to 1:2.0.

Table 5.5. The influence of detergents on the PLD activity. The PLD activity was measured by standard assay (4.9.1.1.) but with different PC-detergent ratios as explained in section 4.9.8. The PC concentration was 5 mM. Data are the mean ± SD obtained from duplicate experiments.

Detergents	**PLD activity in the presence of different molar ratio of PC to detergent (µmol min^{-1} mg^{-1})**					
	1 : 0.25	**1 : 0.5**	**1 : 1.0**	**1 : 1.5**	**1 : 2.0**	**1 : 4.0**
Triton X-100	0.0	0.0	18.8 ± 3.2	46.3 ±16.0	56.3 ± 8.7	54.7 ± 8.8
Tween 20	0.0	n.d	0.0	0.0	0.0	n.d
Brij-58	0.0	n.d	0.0	6.5 ± 8.8	0.0	n.d
SDS	140.1 ± 8.6	252.1 ± 2.4	257.8 ±1.3	208.3 ± 5.2	nd	nd
Sodium deoxycholate	11.8 ± 1.5	153.1 ± 7.8	185.8. ± 3.2	194.8 ± 9.2	134.3 ± 12	nd
CTAB	0.0	0.0	32.0 ± 5.0	35.5 ± 7.3	60.6 ± 4.9	0.0

5.3.8. Effect of Inhibitors on the activity of the PLD

The effect of AlF_4^- and Na_3VO_4 as inhibitors of mustard PLD was studied with artificial substrate P*p*NP as described in section 4.9.9. PLD was pre-incubated with increasing concentrations of AlF_4^- (a combination of 3 mM of NaF and increasing concentration (0-70 µM) of $AlCl_3$) for 5 min before addition to the reaction medium. AlF_4^- potently inhibited PLD enzyme activity as shown in fig 5.16A.

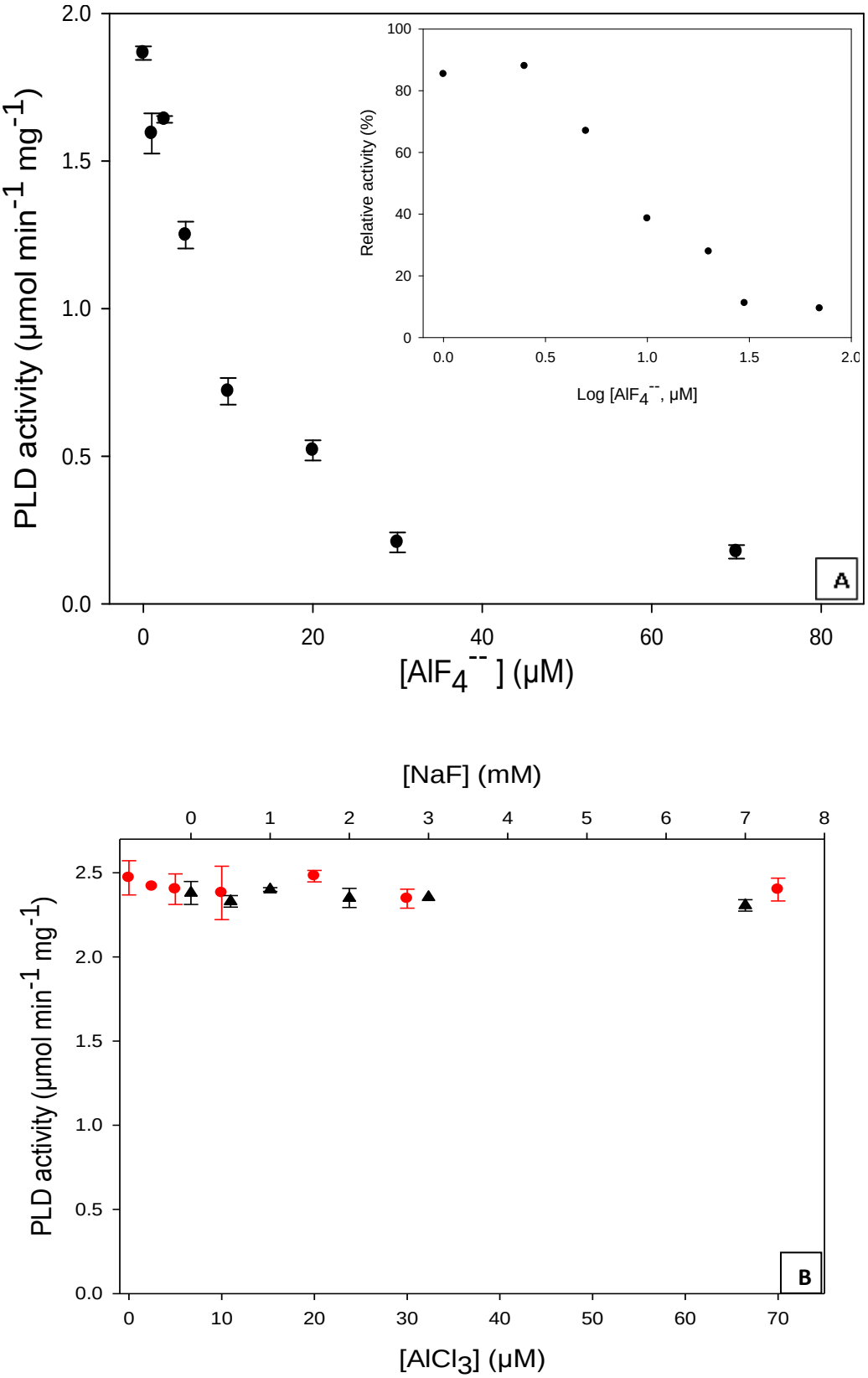


Fig 5.16. **Effect of AlF_4^- on the activity of PLD. (A)** PLD activity as a function of varying concentrations of AlF_4^-. The inset shows relative activity of the PLD against log of AlF_4^-concentrations. The IC_{50} value was derived by fitting the curve with sigmoidal model for the ligand-binding dose response curve (SigmaPlot, version 11.0). **(B)** Individual efects of varying concentrations $AlCl_3$ (red circles) and NaF (black triangles) on the activity of PLD. PLD activity was measured with P*p*Np assay as described in section 4.9.9.

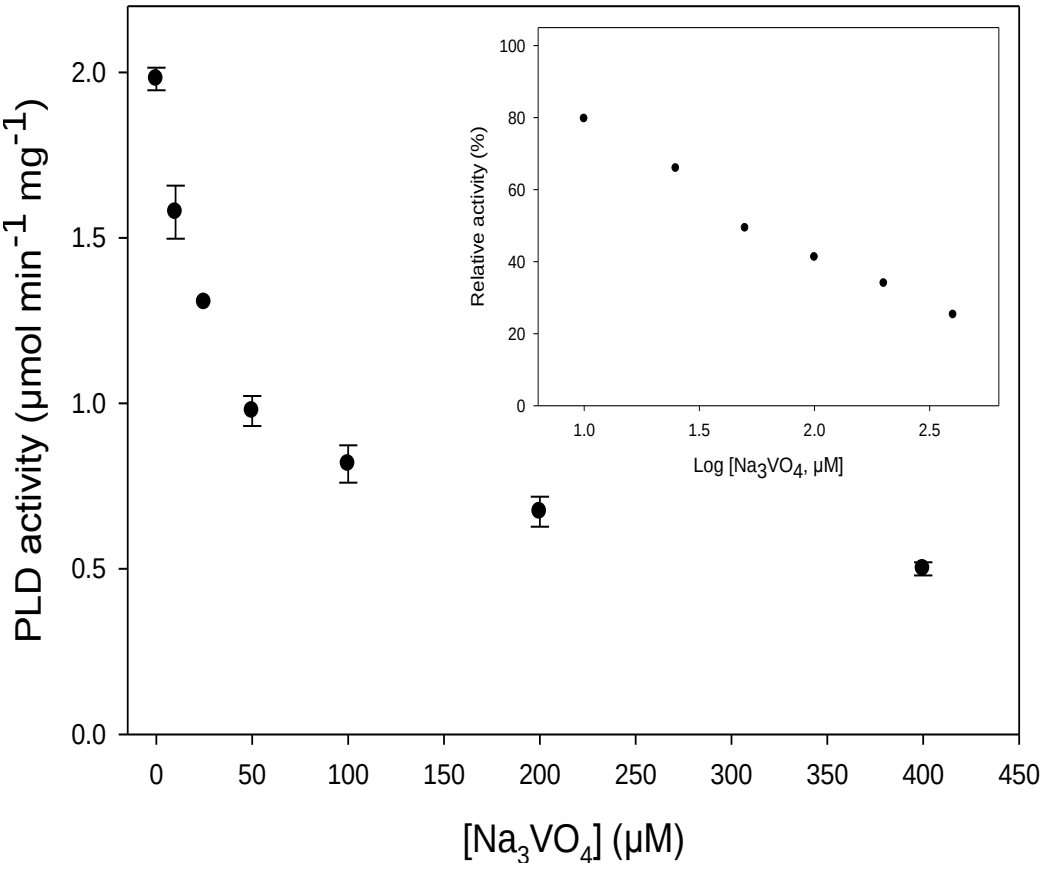


Fig. 5.17 Effect of Na_3VO_4 on the PLD activity. PLD activity as a function of varying concentrations of Na_3VO_4. The inset shows relative activity of the PLD against log of Na_3VO_4 concentrations. The IC_{50} value was derived by fitting the curve with the sigmoidal model for the ligand-binding dose response curve (SigmaPlot software, version 11.0). PLD activity was measured with P*p*NP assay as described in section 4.9.9.

The activity was reduced by more than 75% in the presence of 20 µM AlF_4^- (fig 5.16A). The IC_{50} value of AlF_4^-, calculated for mustard PLD was 7.8 µM as derived from the inset of fig 5.16A. Individual effects of NaF (0-7 mM) and $AlCl_3$ (0-70 µM) on PLD were also investigated and neither of them were found to inhibit PLD enzyme activity within their respective concentration range studied as shown in fig 5.16B. Similarly, fig 5.17 showed the inhibitory effect of Na_3VO_4 on PLD enzyme activity. The IC_{50} value calculated for Na_3VO_4 was 33 µM as derived from the inset of fig 5.17.

5.3.9. Effect of PIP_2 on the PLD activity

As PIP_2 is known to be a regulator of PLD activity, the effect of PIP_2 on the PC hydrolysis as a function of calcium ions (0-10 mM) was investigated as described in section 4.9.9. The activity of the PLD is also presented relative to its activity at optimum $CaCl_2$ concentration of 70 mM as used in the standard assay conditions (described in section 4.9.1.1). At all Ca^{2+} ions

concentration, the PLD activity was higher in the presence of PIP_2 than in its absence (fig 5.18). At low Ca^{2+} ions concentration (in the range of 0.1-1 mM), 45-70% of PC hydrolysis was observed in the presence of PIP_2 as compared to 2-9% in the absence of PIP_2. The role of PIP_2 in the PLD activation was examined by using neomycin, a high affinity ligand that selectively binds to polyphosphoinositides and blocks their binding to their targets (Gabev *et al.*, 1989). Addition of 1 mM neomycin in the reaction mixture resulted in a great reduction of PLD activity (fig 5.18). At Ca^{2+} ions concentration of 0.5 mM, the PLD activity decreases from 60% in the absence of neomycin to less than 30% in its presence. These observations showed that PIP_2 greatly stimulated the activity of mustard PLD when incorporated in PC vesicles even in micromolar amount.

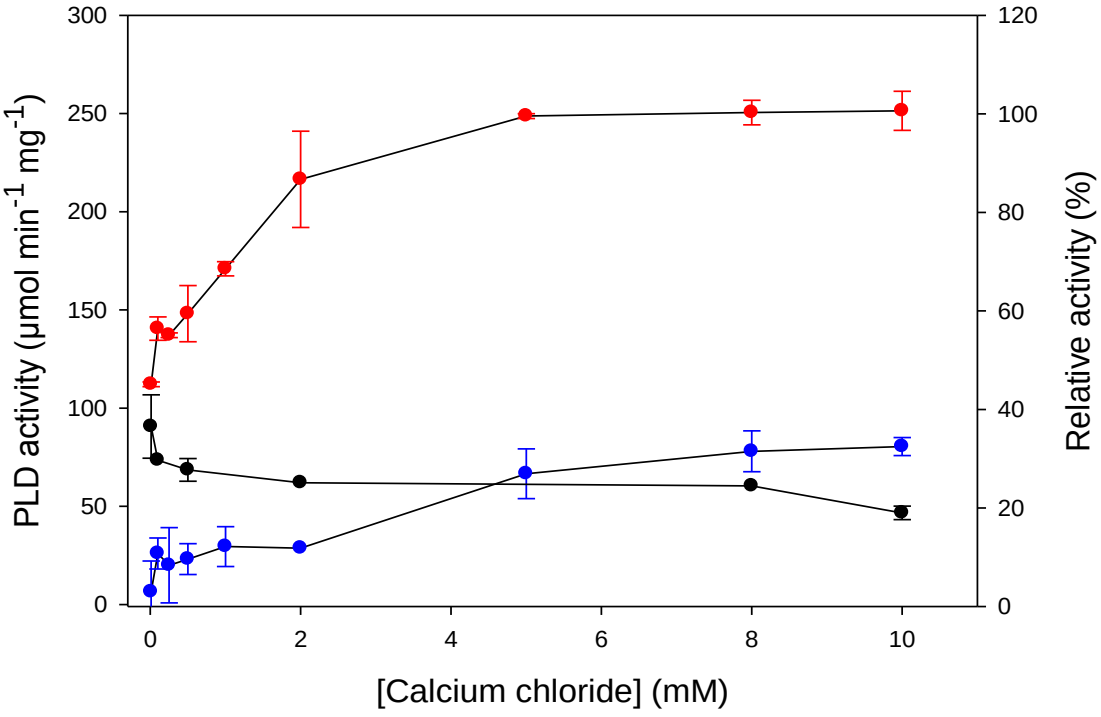


Fig 5.18. Effect of PIP_2 on PC hydrolysis by the PLD as a function of calcium ions concentration. The activity of PLD was calculated relative to its activity at optimum calcium chloride concentration of 70 mM (according to section 4.9.1.1). Red circles shows PLD activity towards PC/PIP_2 vescicles (molar ratio 98:2), blue circles shows the activity in the absence of PIP_2 and black circles shows PLD activity with PC/PIP_2 vescicles in the presence of 1mM neomycin.

5.4. Characterization of the PLD in aqueous-organic two-phase system

5.4.1. Effect of organic solvents on the activity of the PLD

Reactions with PLD can also be performed in two-phase systems. They occur at the interface of aqueous and organic phase, where the organic phase consists of the phospholipid dissolved

in an organic solvent. The effect of organic solvents on the activity of PLD was investigated as described in section 4.10.2. Table 5.6 illustrates the PLD hydrolytic activity in various solvents with respect to its activity in n-hexane/2-octanol (7.8:1) system. The enzyme showed maximum activity when the organic phase used was n-hexane/2-octanol system followed by toluene and methylene dichloride, respectively. Absolutely, no activity was detected when the solvents like n-hexane or chloroform were used.

Table 5.6. Hydrolytic activity of mustard PLD towards PC dissolved in different organic solvents. Relative activity of PLD in different organic solvents with respect to its activity in n-hexane/2-octanol system (7.8:1). The activity was measured as described in section 4.10.2.

Solvents used	Relative activity (%)
n-hexane /2-octanol (7.8:1)	100 ± 1.4
Toluene	23.6 ± 0.7
Methylene chloride	13.5 ± 0.9
Diethyl ether	4.5 ± 0
Chloroform	0.0 ± 0.0
n-hexane	0.0 ± 0.0

As clear from table 5.6, no PLD activity was observed when n-hexane was used as the organic solvent but the inclusion of low amount of aliphatic alcohol (2-octanol) resulted in the highest PLD activity as compared to other solvents studied. Motivated by this effect the influence of other lower secondary alcohols like 2-hexanol and 2-butanol on the hydrolysis of PC was investigated. In addition to this the effect of 1-octanol was compared to that of 2-octanol on PLD activity. No transphosphatidylation product was detected in the presence of these alcohols. Fig 5.19 depicts the initial rate of PC hydrolysis as a function of increasing concentration of additive alcohols. As clear from fig 5.19, the PLD activity in the n-hexane/buffer system is strongly elevated in the presence of small amount of aliphatic

alcohols. The activating effect depends upon the concentration as well as on the chain length of the alcohols. At the optimal concentration of alcohols, 2-octanol has the greatest enhancing effect followed by 1-octanol and 2-hexanol and the least effect was observed with 2-butanol. For each alcohol, the rate of hydrolysis increases with increasing concentration of alcohol and reaches a plateau or decrease beyond the optimum. The optimum concentrations of alcohols in the reaction mixtures were in the range of 0.1-0.4 mmol, which corresponds approximately to a molar excess of 80-320**.**

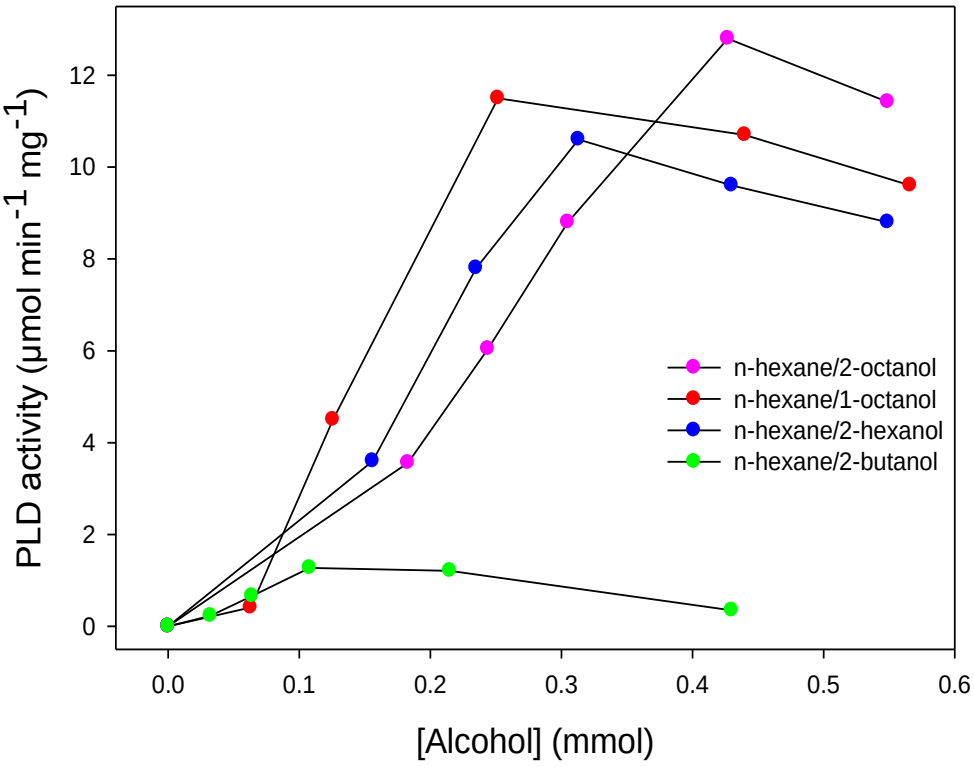


Fig 5.19. Activity of PLD in n-hexane/buffer system containing various aliphatic alcohols. PLD activity was assayed in n-hexane/buffer system containing varying amount of aliphatic alcohols as described in section 4.10.2. The experiments were conducted in duplicates and the variations were less than 6%.

5.4.2. Effect of pH on the activity of the PLD

The effect of pH on the hydrolytic activity of mustard PLD was studied as described in section 4.10.3. As seen in fig 5.20, the hydrolytic activity of mustard PLD increases with increasing pH from pH 4.25 to 5.5 after which it started to decline slowly.

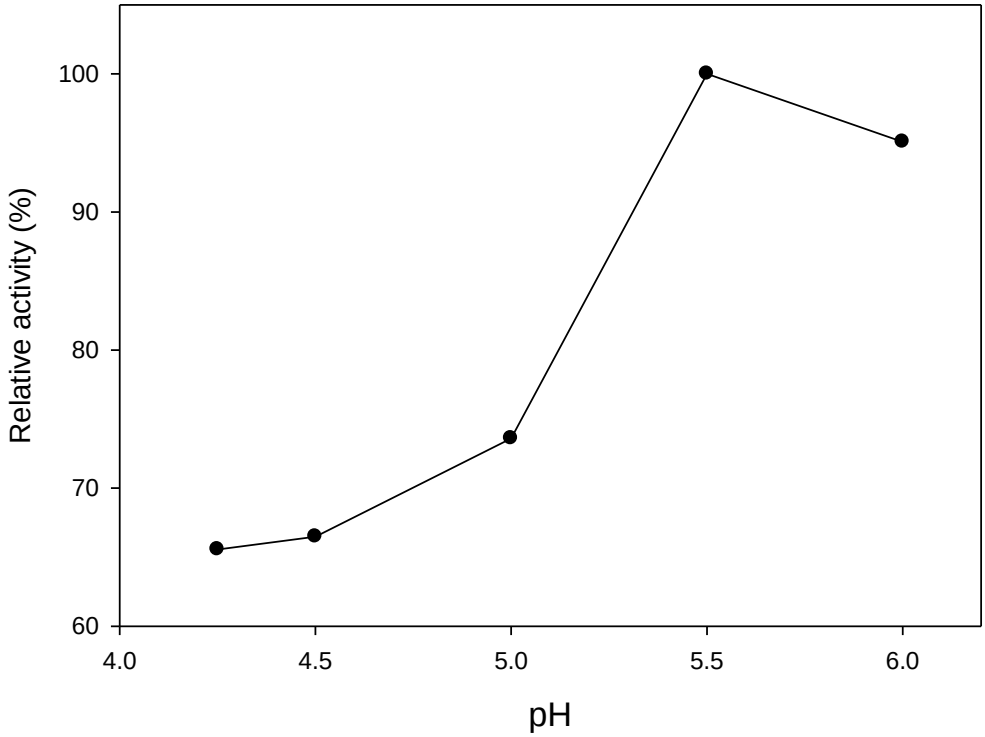


Fig. 5.20. Effect of pH on hydrolytic activity of mustard PLD. PLD activity was analyzed with the standard assay but the buffers used were 0.3 M sodium acetate buffer, pH 4.25-6.0 containing 120 mM calcium chloride as described in section 4.10.3.

5.4.3. Effect of Ca^{2+} ions on the activity of the PLD

The effect of calcium ions on the hydrolytic activity of PLD was investigated as described in section 4.10.4. Fig 5.21 demonstrated a plot of the initial rate of hydrolysis (as relative activity) against the increasing concentration of calcium ions. Calcium was absolutely required for PLD activity and no activity was detected in the absence of calcium ions (fig 5.21). With the increase in calcium ions concentration, the PLD activity increases and reaches a plateau at 50 mM and then remained constant till 120 mM. The apparent binding constant for Ca^{2+} ions (K_{ca}) was calculated by the fitting of the curve with one site saturation/ligand binding equation (SigmaPlot, version 11.0) and was found to be 17.8 ± 4.5 mM.

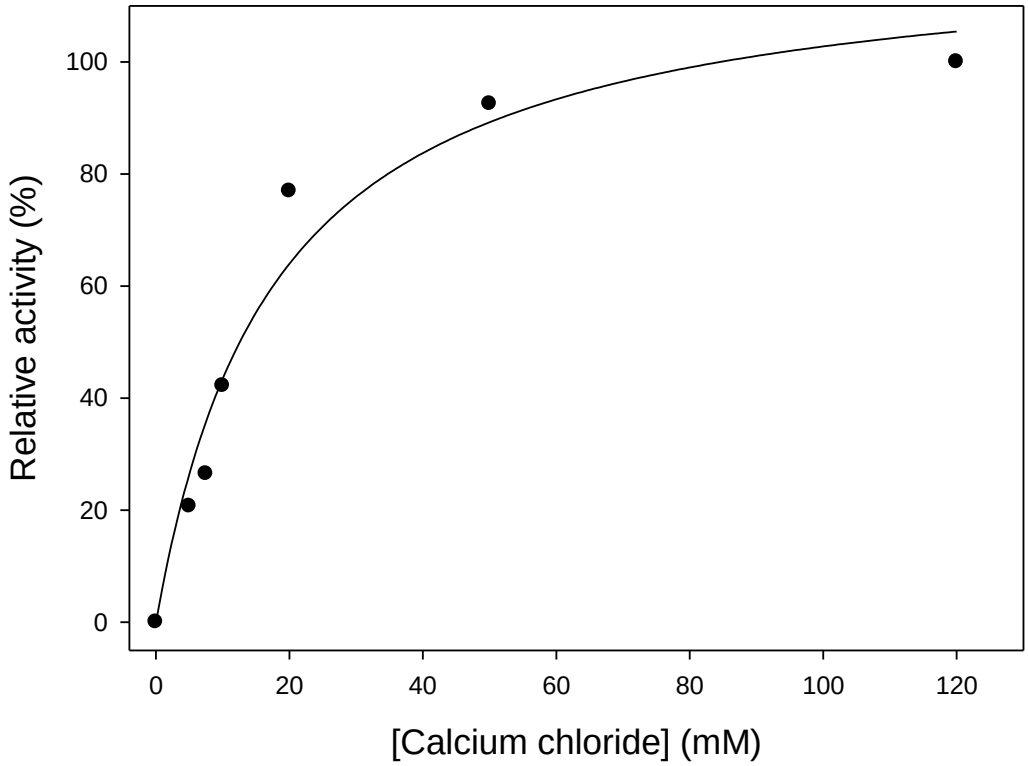


Fig 5.21. Effect of the calcium ions on the activity of PLD. PLD activity was assayed in 0.3 M sodium acetate buffer containing varying concentrations of calcium chloride (0-120 mM) as described in section 4.10.4.

5.4.4. Kinetic parameters of the PLD

The effect of substrate concentration on the activity of PLD was investigated in aqueous-organic two-phase system using soya PC as substrate (described in section 4.10.5). The initial rate of hydrolysis was plotted as a function of substrate concentration as shown in fig 5.22. PLD activity increases hyperbolically with the increase in substrate concentration. The fitting of the curve was performed using Michaelis-Menten equation and the inset (fig 5.22) showed the Lineweaver Burk plot of the data obtained from fig 5.22A. The values of V_{max} and K_m as calculated were 16.9 ± 1.8 µmol min^{-1} mg^{-1} and 0.96 ± 0.25 mM, respectively.

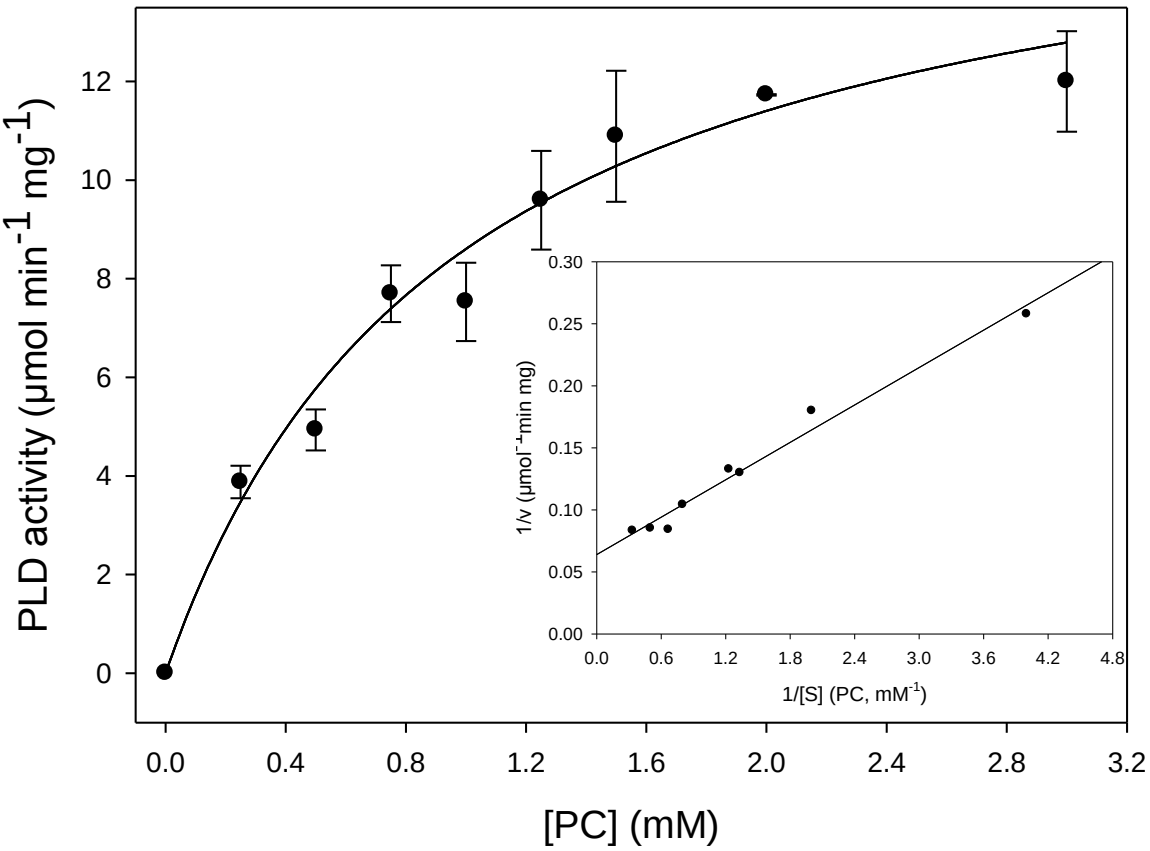


Fig 5.22. Effect of substrate concentration on the PLD activity. The initial rates of PC hydrolysis is plotted as a function of substrate concentration (0-3 mM). The inset showed the Lineweaver-Burk plot of the data. The activity was measured by the assay conditions described in section 4.10.5.

5.4.5. Substrate specificity of the PLD

The substrate specificity of mustard PLD with naturally occurring substrates was investigated as described in section 4.10.6. The table 5.7 showed the initial rates of hydrolysis of phosphatides with different alcoholic head groups, relative to its activity towards PC. The enzyme possesses highest hydrolytic activity with PC followed by PE and PG in the following order PC > PE > PG. A very small but significant amount of hydrolytic activity was also detected with PI. However, no hydrolysis was observed with PS even after 150 min of reaction time.

Table. 5.7. Substrate specificity of the PLD. Initial rates of hydrolysis of phosphatides with different alcoholic head groups. The activity of the PLD with other substrates was presented as relative to its activity with PC. The activity was measured using the assay conditions as described in section 4.10.6. Data are the mean ± SD obtained from duplicate experiments.

Phospholipids	Hydrolytic activity (µmol min^{-1} mg^{-1})	Relative activity (%)
Phosphatidylcholine	10.4 ± 1.2	100
Phosphatidylethanolamine	8.94 ± 0.28	86
Phosphatidylglycerol	1.45 ± 0.36	14
Phosphatidylserine	0	0
Phosphtidylinositol	0.016 ± 0.0	0.15

5.4.6. Transphosphatidylation activity of the PLD

The transphosphatidylation activity of the mustard PLD in competition to hydrolysis was investigated in the presence of various natural alcohols i.e. ethanolamine, glycerol, myo-inositol, serine and glycol taking PC as the substrate (described in section 4.10.1). Activity analysis in the absence of alcohol was also performed as a reference. Table 5.8, showed the initial rate of hydrolysis and transphosphatidylation in the presence of respective acceptor alcohols (1.2 M). The transphosphatidylation selectivity ($v_T/(v_T + v_H)$ for each alcohol is presented as a ratio of tansphosphatidylation activity to the total activity in the presence of that alcohol. The transphosphatidylation potential (v_T/v_H) of the mustard PLD in competition to hydrolysis is demonstrated as a ratio of initial rate of tranphosphatidylation to initial rate of hydrolysis. As described in section 2.3.2.5, the transphosphatidylation potential or the transphosphatidylation selectivity, respectively are the decisive parameter for the catalytic efficiency of a PLD. Mustard PLD showed highest transphosphatidylation potential with ethanolamine (5.8) followed by glycerol (3.6) and glycol (2.4). Mustard PLD also accepted L-serine as one of the acceptor alcohol although with low conversion rate of 0.22 (table 5.8).

Table. 5.8. Transphosphatidylation activity of the PLD. Initial rates of hydrolysis (V_H) and transphosphatidylation (V_T) in the presence of various acceptor alcohols. Transphosphatidylation selectivity and potential are presented as V_T /(V_H + V_T) and **(**V_T / V_H), respectively. The reactions were performed and analyzed using the standard assay conditions as described in section 4.10.1. Data are the mean ± SD obtained from duplicate experiments.

Acceptor alcohols	**V_H (µmol min^{-1} mg^{-1})**	**V_T (µmol min^{-1} mg^{-1})**	**V_T /(V_H + V_T)**	**V_T / V_H**
Without alcohol	10.8 ± 1.44	0.0	0.0	0.0
Ethanolamine	0.77 ± 0.03	4.43 ± 0.02	0.85	5.8
Glycerol	0.65 ± 0.1	2.31 ± 0.03	0.78	3.6
L-serine	1.26 ± 0.16	0.28 ± 0.02	0.18	0.22
Myo-inositol	2.07 ± 0.12	0.0 ± 0.0	0.0	0
Glycol	0.52 ± 0.11	1.26 ± 0.02	0.71	2.4

6. Discussion

6.1 Purification of PLD from mustard seeds

PLD activity with some uncommon properties had been identified in the crude extract of dry mustard seeds (Khatoon *et al.,* 2007). In the present study we were successful in characterizing this enzyme in its purified form.

To the best of our knowledge this study presents the first report on the purification of PLD from mustard seeds. Purification steps like ammonium sulphate precipitation, calcium-mediated hydrophobic interaction chromatography on Octyl-Sepharose CL-4B and anion exchange chromatography on Source 15Q, which are commonly employed for the purification of most plant PLDs also proved successful in the present study (section 5.1; fig 5.1, 5.2 and table 5.1), and resulted in a homogenous preparation of mustard PLD (section 5.1; fig 5.3).

Interestingly, Ca^{2+} ions-mediated hydrophobic interaction chromatography on Octyl-Sepharose CL-4B had to be operated at room temperature because previous attempts to carry out the process under cold conditions (4-5^{o}C), as used in the standard protocols, were unsuccessful (section 5.1). The binding of PLD to the hydrophobic matrix in the presence of calcium ions is proposed to be mediated through hydrophobic interactions (Lambrecht and Ulrich-Hofmann, 1992). Since, it is well known that the hydrophobic forces are enhanced by high temperature and ionic strength, thus it is quite possible that at lower temperatures (4-5°C), the hydrophobic interactions between PLD enzyme and chromatographic matrix were not sufficient enough to contribute to the binding of enzyme to the matrix while at relatively higher temperature (room temperature of around 23°C) these hydrophobic interactions got strengthened and therefore resulted in the strong binding of enzyme to the matrix. Similar observation was also made with the purification of calmodulin on Phenyl-Sepharose matrix in the presence of calcium ions (Gopalakrishna and Anderson 1982). In another example, binding of haptaglobin on the hydrophobic matrix of Cibacron Blue F3-GA at 25°C and not at 4°C, further enforces the influence of temperature on hydrophobic interaction chromatography (Gianazza and Arnaud, 1982).

6.2 Molecular characterization

Molecular mass of the mustard PLD was estimated as 91.9 ± 1.9 kDa (section 5.2.1, fig 5.4), which is in the range of molecular masses of other plant PLDs as obtained by the method of SDS-PAGE. Thus, molecular masses between 87 and 90 kDa were reported for PLD from

cabbage (Lambrecht and Ulbrich-Hofmann, 1992; Abousalham *et al*., 1993), 92 kDa for PLD from sunflower seeds (Abousalham *et al.,* 1997), 92 kDa for PLDs from castor bean (Wang *et al.,* 1993) and peanut seeds (Nakazawa *et al.,* 2006). Also, the pI value for the mustard PLD, which was observed in the range of 4.3-4.4 (section 5.2.2; fig 5.6A), falls in the acidic range as for other plant PLDs. For instance it is 4.2 for castor bean PLD (Wang *et al.,* 1993), 4.7 for cabbage PLD (Schöps *et al.,* 2002), 4.7 for soybean PLD (Abousalham *et al.,* 1995). The enzyme also displayed cross-reactivity with the antibodies raised against poppy PLDα1 (section 5.2.2; fig 5.6B), which suggests that the two proteins are antigenically similar i.e the protein portions that resulted in synthesis of antibody are similar. Such antigenic relationship was also observed between PLDs extracted from castor bean, cabbage, peanuts, barley and the antibodies raised against castor bean PLD (Wang *et al*., 1994). In addition to this, the peptides obtained from mustard PLD after tryptic digestion followed by mass spectrometry demonstrate a strong sequence identity with other plant PLDs specifically to the α-types (section 5.2.3; fig 5.7, table 5.2). The above observations indicate that mustard PLD possess characteristics similar to other plant PLDs in particular to α-type PLDs.

6.3 Kinetic characterization

As valid for lipolytic enzymes in general, PLDs are more active to substrate molecules in aggregated than in monomeric form, which is demonstrated by the typical increase of activity to micelle-forming substrates above the critical micelle concentration (Allgyer and Wells, 1979; Lambrecht and Ulbrich-Hofmann, 1992; Abousalham *et al.,* 1997; Kuppe *et al.,* 2008). As explained in section 2.1, the two techniques which are successfully employed for the quantitative and reproducible kinetic measurements of such lipolytic enzymes, include the use of either detergents or organic solvents in substrate formulation. Water immiscible long chain phospholipids substrates are easily solubilized in these components. They get transformed into an optically clear solution of micelles or reverse micelles, which are readily accessible for enzyme attack.

Thus, kinetic characterization of the mustard PLD was performed both in the mixed micellar as well as in aqueous-organic two-phase system. In the mixed micellar system containing PC/SDS/Triton X-100 (1: 0.5: 1.6) as well as in the aqueous-organic two-phase system with n-hexane/2-octanol (8:1) as organic solvent, the enzyme displayed a hyperbolic characteristic of v/[S] curves (section 5.3.1; fig 5.8 and section 5.4.4; fig 5.22). The corresponding V_{max} and K_m values for mustard PLD in mixed micellar system were 356 ± 15 $\mu molmin^{-1}mg^{-1}$ and 1.84

± 0.17 mM respectively. PLDs from other plant sources such as cabbage (Lambrecht and Ulbrich-Hofmann, 1992) and peanut (Abousalham *et al.,* 1999) also revealed hyperbolic v/[S] curve, when their activity was measured in a mixed micellar system containing long chain natural substrate (PC) and detergents in a definite proportion. The corresponding V_{max} and K_m values for cabbage PLDα2 calculated towards egg PC-SDS (1:2) mixed micelles were 102.5 µmol min^{-1} mg^{-1} and 0.1 mM respectively at pH 5.6 (Lambrecht and Ulbrich-Hofmann, 1992) and for peanut PLD towards egg PC-sodium deoxycholate mixed micelles were 32.64 µmol min^{-1} mg^{-1} and 0.072 mM respectively at pH 5.0 (Abousalham *et al.,* 1999). The related kinetic parameters for the mustard PLD as calculated in aqueous-organic two-phase system were V_{max} = 16.9 ± 1.8 µmol min^{-1} mg^{-1} and K_m = 0.96 ± 0.25 mM, i.e., they differed from the corresponding parameters obtained in aqueous micellar system by a factor of 2 and 21 respectively. This supports the great importance of the substrate presentation for evaluation of the kinetic behaviour, which must be considered in the comparison of different enzymes and also in their application.

Mustard PLD displayed optimum activity in an acidic range in both the mixed micellar (section 5.3.2; fig 5.9A) as well as aqueous-organic two phase assay system (section 5.4.2; fig 5.20). In mixed micellar assay system, the optimum activity in the acidic pH range was also observed for PLDs from other plant sources such as tomato PLD (Jandus *et al.,* 1997), rape seed PLD (Novatna *et al.,* 1999), PLD-A from poppy (Oblozinsky *et al.,* 2003), PLD1 and PLD2 from cabbage (Schaeffner *et al.,* 2002), PLD1 and PLD2 from poppy (Lerchner *et al.,* 2005) and peanut PLD (Nakazawa *et al.,* 2006). Likewise, in aqueous-organic two-phase system, the pH optimum in the acidic range was also observed for partially purified cabbage PLD (Hirche *et al.,* 1999), poppy PLD-A (Oblozinsky *et al.,* 2003). The enzyme exhibited moderate stability in a relatively broad pH range (pH 5.0-9.0) (section 5.3.2; fig 5.9B). Despite its wide range, the pH of optimal stability did not coincide with the pH of optimal activity but the enzyme showed relatively lesser stability (~ 50%) in the pH range (pH 5.5-6.0) where it was maximally active (section 5.3.2). Similar observations were made with cabbage and cotton seed PLD (Harvey and Balls 1955).

A relatively steep temperature optimum of around 45°C was observed for mustard PLD, when its activity was assessed in mixed micellar system (section 5.3.3; fig 5.10A). PLD from other plant sources exhibited optimum activity in the similar temperature range for instance 40°C for tomato PLD (Jandus *et al.,* 1997). The activation energy of mustard PLD, calculated in the

temperature range of 25-45°C was 8.8 kcal mol^{-1} (section 5.3.3; fig 5.10B), which is similar to the activation energy of 9.1 kcal mol^{-1} as reported for peanut PLD (Heller *et al.,* 1976). Besides, the purified PLD displayed a retention of less than 10% relative activity when exposed to 55°C for 8 min at pH 7.0 (section 5.3.3; fig 5.11). This result is in accordance with the moderate thermal stability of PLDs from other plant sources like cabbage PLD (Stumpe *et al.,* 2007) but in contradiction to the previous study made with the crude preparation of mustard seeds, where the enzyme showed high thermal stability (Khatoon *et al.,* 2007). The reason for this contrasting results might be due to a stabilizing protein or other component in the crude extract of the mustard seeds which become lost during purification.

6.4 Effect of metal ions, organic solvents, inhibitors and PIP_2

Activating and destabilizing effects of Ca^{2+} ions

Mustard PLD displayed an absolute requirement of metal ions for activity (section 5.3.4; table 5.3) with a strong preference for Ca^{2+} ions. Low but significant enzyme activity was also observed in the presence of magnesium, zinc and manganese ions while no activity was detected in the presence of potassium, nickel or lithium ions. These observations are in contradiction to those observed with the study on crude extract of mustard seeds (Khatoon *et al.,* 2007), where the addition of metal ions was not mandatory for the enzyme activity. Probably the crude extract contained calcium ions in concentration that were not completely complexed even in the presence of 1.5 mM EDTA (Khatoon *et al.,* 2007).

The calcium ions concentration required for optimum activity in mixed micellar and two-phase system was similar, 60 mM (section 5.3.5; fig 5.13) and 50 mM (section 5.4.3; fig 5.21), respectively showing that the activation effect is more induced by the binding of the Ca^{2+} ions to the enzyme than to the substrate. The requirement of Ca^{2+} ions in millimolar range for optimal activity in both the assay systems affirmed that mustard PLD belongs to the group of α-type PLDs.

From the activity curve as a function of Ca^{2+} ions concentration, two binding events with apparent dissociation constants, K_{D1} = 0.146 mM and K_{D2} = 16.1 mM were derived (section 5.3.5; fig 5.13). Similarly Stumpe *et al.* (2007) derived two binding sites with K_{D1} = 0.073 mM and K_{D2} = 17.1 mM from activity measurements of PLDα2 from cabbage.

At a Ca^{2+} ion concentrations above 60 mM to up to 200 mM, the activity of mustard PLD remained constant (section 5.3.5; fig 5.13). This finding was similar to the one observed with tomato PLD whose activity (in the presence of 0.05% SDS) reaches its maximum at 80 mM

of calcium ions concentration and then remain constant on further increase of calcium ions in the reaction mixture till 200 mM (Jandus *et al.*, 1997). However, with other PLDs this behaviour was different such as with cabbage PLDα2 whose activity decreases as the calcium ion concentration in the reaction mixture increases beyond 100 mM (Stümpe *et al.*, 2007) or with PLD1 and PLD2 from poppy whose activity continued to increase with the calcium ion concentration up to 200 mM (Lerchner *et al.*, 2005). Furthermore, no shift in pH optimum was observed for mustard PLD when Ca^{2+} ions concentration was varied from 0-70 mM (section 5.3.5; fig 5.14). Similar observation was made with the recombinantly expressed PLDα1 and α2 from cabbage where also no shift in pH optima was obtained within the range of 0-100 mM Ca^{2+} ions concentration (Schaeffner *et al.*, 2002) Unlike to this observation, a strong influence of Ca^{2+} ions concentration on the pH optima of PLDs from different plant sources has been reported in literature (discussed in section 2.2.2.3).

Metal ions though required for the mustard PLD activity, exerted a destabilizing effect on its stability. As shown in section 5.3.4 (fig 5.12), the destabilizing effect of salts was independent of the type of ions, when compared at the same ionic strength. A Similar observation was made with cabbage PLDα2 by Stumpe *et al.* in 2007. It was proposed in their study that purified cabbage PLDα2 has a strong tendency to aggregate and this aggregation propensity is enhanced because of high salt concentration which in turn resulted from increased hydrophobic interactions.

PIP_2 as an activator of the PLD activity

PIP_2 acts as a regulator of the mustard PLD as the enzyme activity was greatly increased even at micromolar Ca^{2+} ions concentration, when PIP_2 (0.02%) was incorporated into the PC vesicles (section 5.3.9; fig 5.18). PIP_2 has been shown to be a specific activator molecule of PLD in mammalian as well in plant sources (discussed in section 2.2.2.6). Although, not essential for the activity of α-type PLDs, it can stimulate their activity (Qin *et al.*, 1997; Pappan and Wang 1999). It was revealed that α-type PLDs can be active at micromolar concentrations of Ca^{2+} ions under acidic conditions (pH 4.5-5.5), if PIP_2 or phosphatidylinositol 4-phosphate is present in the lipid vesicles (Pappan *et al.*, 1998). Again, in the previous study with the dialyzed crude extract of mustard seeds, it was found that PIP_2 did not act as an activator of the PLD activity (Khatoon *et al.*, 2007). However, in that study, PIP_2 was not incorporated in the substrate vesicles but added exogenously into the reaction medium.

Detergents as stimulators of the PLD activity

The activity of mustard PLD was strongly stimulated by detergents (section 5.3.7; table 5.5). In general, anionic detergents (SDS and sodium deoxycholate) had the strongest stimulating effect on the enzyme activity followed by Triton X-100 and CTAB. PLD from other sources are also stimulated by the anionic detergents for instance tomato PLD showed enhancement in activity when the SDS concentration in the reaction mixture was increased from 0.05% (m/m) to 0.35% (m/m). The same enzyme also showed absolute requirement of Triton X-100 in substrate vesicles for activity (Jandus *et al.,* 1997). The activation effect of SDS was also observed with PLDs from other plant sources such as rape (Novatna *et al.,* 1999) and cabbage (Lambrecht and Ulbrich-Hofmann, 1992). The stimulating effect of detergents was greatly influenced by the molar ratio of PC to detergent used in the substrate solution. With the increase in the molar ratio of PC to detergent, the enzyme activity increased, reached a plateau and then slowly began to decline (section 5.3.7; table 5.5). Dennis in 1973, has performed kinetic studies on a PLA2 from cobra venom with long chain PC in the presence of Triton X-100. He proposed that the decrease in enzyme activity with increasing relative detergent concentration may be attributed to the decreasing phospholipid density/unit surface area (or surface dilution of the substrate) with the assumption that enzyme binds strongly to the mixed micelles irrespective of its chemical structure. Abousalham et al. (1997) also studied PLD from germinating sunflower seeds in mixed micellar (egg PC/SDS/Triton X-100) system with variable detergent/phospholipid ratios. They observed that optimum PLD activity was obtained at PC/detergent molar ratio of 1:1.5, after which it started to decrease. It was suggested that below this ratio a part of the substrate probably forms bilayer structure or insoluble phases which may not be available for the enzyme attack. Moreover, above this ratio all of the substrate was present in micellar form which got diluted by further addition of detergents and thus leads to the decrease in enzyme activity.

Effect of phosphate analogs as inhibitors of the PLD activity

Phosphate analogs (AlF_4^- and Na_3VO_4) demonstrated a strong inhibitory effect on mustard PLD activity with the IC_{50} values of 7.8 and 33 µM respectively (section 5.3.8; fig 5.16A and 5.17). Similarly, Li and Flemming (1999a) reported the inhibitory effect of phosphate analogs like AlF_4^-, Beryllium fluoride (a combination of 50 µM $BeCl_3$ and 3 mM NaF), Na_3VO_4 and inorganic phosphate on cabbage PLD. In their study, the IC_{50} values of AlF_4^- and Na_3VO_4 for cabbage PLD was found to be 15 and 33 µM, respectively. They also observed the inhibitory effect of AlF_4^- on peanut PLD and rat PLD (Li and Flemming 1999a) and suggested that the

mechanism of these inhibitors was based on their phosphate mimicking property. Since, it is known that the PLD catalyzed reaction proceeds via phosphohistidine linked phosphatidyl-enzyme intermediate (Gottlin *et al.*, 1998, Rudolph *et al.*, 1999 and Leiros *et al.*, 2004), AlF_4^- or other phosphate analogs might bind to the phosphate binding site of the catalytic domain of PLD (possibly to a His residue) and thus prevent the formation of a phosphatidyl-enzyme intermediate. The present results on mustard PLD supported the idea that the phosphate analogs like AlF_4^- are the universal inhibitor of PLD in both plant and animal system.

Effect of organic solvents on the PLD activity in two-phase assay system

In two-phase systems the kind of organic solvents was extremely important for the activity of mustard PLD. The highest rate of PC hydrolysis was observed in n-hexane/2-octanol (8:1) system followed by toluene, methylene dichloride and diethyl ether (section 5.4.1; table 5.6). No activity was observed when chloroform or n-hexane was used as organic solvent. Some of these findings were in close accordance to the observations made for cabbage PLD (Hirche and Ulbrich-Hofmann, 1999), where also absolutely no activity was observed in n-hexane and a very low PLD activity was observed in chloroform. Contrarily, the mustard PLD exhibited very low activity when diethyl ether was used as an organic solvent which is unlikely to PLDs from other plant sources such as cabbage which exhibited highest activity in diethyl ether (Hirche and Ulbrich-Hofmann, 1999). As discussed above, no PC hydrolysis was observed with pure n-hexane/buffer system, however, an addition of small amount of aliphatic alcohols resulted in a great rise in hydrolytic activity of mustard PLD (section 5.4.1; fig 5.19). The stimulating effect of additive alcohols on the activity of the mustard PLD followed the given order: 2-octanol > 1-octanol > 2-hexanol > 2-butanol. Similar observations were made by Hirche et al. (1997) with partially purified cabbage PLD. It was analyzed in this study that the major part of the enzymatic conversion occurs at the interphase of the aqueous-organic phase and that the conversion rate is strongly influenced by the structure and the packing density of the PC molecules at the interface. In case of strongly hydrophobic solvents like n-hexane, the packing density is too high for efficient catalysis but when alcohols are added (as in case of n-hexane/alcohol) the packing density become loosened. This contributes to the formation of suitable aggregates and hence resulted in the activation of enzyme.

6.5 Substrate specificity and transphosphatidylation potential

The position and composition of fatty acyl chain as well as alcoholic head group of phospholipid substrate has a great effect on the of mustard PLD (section 5.3.6; fig 5.15). PC molecules with short fatty acyl chain lengths (C8-C10) were better substrates than those having longer chain lengths (C12-C20). Moreover, PC molecules with unsaturated fatty acids were hydrolyzed better than saturated ones and the preference depends upon the fatty acid chain length (section 5.3.6; fig 5.15). Although, PLD catalyzes the hydrolysis of phosphodiester bonds and these reactions mainly involved the phospholipid polar head group, the present data clearly indicate that the specificity of mustard PLD also depend on the fatty acid composition of the PC species. Similar observations were also made with PLD from other plant sources such as soybean PLD (Abdelkafi and Abousalham, 2011), recombinantly expressed cabbage PLDα1/α2 and poppy PLDα1/α2 (Dippe and Ulbrich-Hofmann, 2009b), poppy PLD-A and PLD-B (Oblozinsky *et al.*, 2005). Additionally, mustard PLD displayed no activity towards soya Lyso PC. Low activity with lyso PC has also been reported for other plant PLDs: sunflower PLD (Abousalham *et al.*, 1997) and cabbage PLD (Virto *et al.*, 2000) and also from bacterial PLD from *Streptomyces antibioticus* (Shimbo *et al.*, 1993). Mustard PLD showed no hydrolysis of 1,3 PC molecules consisting of fatty acid chain length of 12 and 14 carbon atoms. Obviously, the structural geometry of these phospholipid molecules does not adapt to the geometry of the active site of the PLD. Interestingly, however, mustard PLD exhibited low but significant activity towards 1,3-8:0/8:0 PC (fig 5.15). This observation is in contradiction to the one with cabbage PLDα2, which displayed absolutely no activity towards 1,3 PC molecules of any fatty acid chain length (Dittrich *et al.*, 1998).

With respect to the head group specificity of phospholipid substrate, PC emerges as the best substrate for mustard PLD followed by PE and PG in both the mixed micellar system as well as two-phase system, however, the level of hydrolysis of each phospholipid varied in both the assay systems. (section 5.3.6; table 5.4 and section 5.4.5; table 5.7). The hydrolytic activity of PLD towards PG (relative to soya PC) was similar in both the two-phase as well as mixed micellar assay system i.e 14 and 17% respectively, however, the activity towards PE was higher in two-phase system (86%) as compared to micellar system (46%). This difference in the relative activities of PE in the two assay systems clearly shows that the alteration in the physical state of substrate affect the level with which the given substrate is hydrolyzed by PLD. Furthermore, PLD enzymes from different plant sources display different preferences towards phospholipid head group (section 2.2.2.7), when studied under different conditions of Ca^{2+} ions, pH and phospholipid composition. For instance cabbage PLDα2, when studied in

mixed micellar system of soya PC/Triton X-100 (1:1) and at similar concentration of Ca^{2+} ions (60 mM), showed highest activity towards PC (82.3 µmol min^{-1} mg^{-1}) followed by PG (27.2 µmol min^{-1} mg^{-1}), while PE (3.1 µmol min^{-1} mg^{-1}) was the least hydrolyzed substrate (Dippe and Ulbrich-Hofmann, 2009a). This data clearly demonstrates that the level of hydrolysis of mustard PLD towards mixed micelles of PC, PG and PE is higher as compared to cabbage PLDα2 at similar Ca^{2+} ions concentration (for reference to mustard PLD activity refer to table 5.4).

Also, as typical of PLDs, mustard PLD also possesses transphosphatidylation activity. It was able to convert PC into PE, PG, phosphatidylglycol and PS in the presence of respective alcohols, with the following order of preference PE > PG > phosphatidylglycol > PS (section 5.4.6; table 5.8). No transphosphatidylation activity was observed in the presence of *myo*-inositol, which is also observed for PLDs from other plant sources. In all cases, hydrolysis of PC was observed along with its conversion to respective phosphatides with the least in case of glycol. Glycerol and glycol showed same conversion rates and it is obvious because of their structural similarity. Interestingly, the enzyme showed conversion of PC to PS but was not able to hydrolyzed PS (table 5.7). Similar observation was also made with two PLD isozymes isolated from poppy which are able to catalyze the conversion of PC to PE but cannot cleave PE (Oblozinsky *et al.*, 2005). It was proposed, that the head group of the phosphatidyl donor and acceptor alcohol occupy different binding sites in the active site of PLD.

7. Summary

The present study describes the purification and characterization of PLD enzyme activity from Indian mustard seeds. The enzyme activity is analyzed towards soya PC in the mixed micellar (PC:SDS:Triton X-100 /1:0.5:1.6) and hexane/2-octanol (8:1) aqueous-organic two phase system, respectively. Following conclusions are withdrawn from the research work carried out:

1. To the best of our knowledge this is the first report on the purification of PLD from mustard seeds. PLD has been successfully purified to apparent homogeneity from the delipidized crude extract of dry Indian mustard seeds with an overall yield of 14% and a specific activity of 254 µmol min^{-1} mg^{-1} towards substrate PC as determined by standard assay in mixed micellar system. The purification procedure includes three steps of ammonium sulphate fractionation, Ca^{2+}-mediated hydrophobic interaction chromatography on Octyl-Sepharose CL-4B and anion exchange chromatography on Source 15Q. The crucial step in the purification of the enzyme is the Ca^{2+}-mediated hydrophobic interaction chromatography on Octyl-Sepharose CL-4B which was performed at room temperature unlike the standard protocol in which the purification step was performed at lower temperature around 4-5°C. The higher temperature was necessary for the binding of PLD on Octyl-Sepharose CL-4B matrix mediated by Ca^{2+} ions.

2. The molecular mass of mustard PLD is estimated as 91.9 ± 1.9 kDa and its pI value is in the acidic range of 4.3-4.4.

3. A high sequence identity of the amino acid sequences of mustard PLD obtained from mass spectrometry analysis to α-type PLDs from other plant sources and the cross reactivity of mustard PLD with anti-poppy PLDα1 disclosed that the enzyme has structural regions similar to PLDs from other plant sources specifically to α-type plant PLDs.

4. Metal ions, preferably Ca^{2+} ions, are mandatory for the PLD activity. The requirement of millimolar levels of Ca^{2+} ions for maximum activity (both in the mixed micellar as well as in two phase system) clearly indicates that mustard PLD belongs to the group of α-type PLDs. Moreover, it is concluded from the activity data as a function of Ca^{2+} ions concentration that calcium ions activate the enzyme in two steps (corresponding to two dissociation constants K_{D1} = 0.146 mM and K_{D2} = 16.1 mM) as a result of the specific binding of the ions at two sites of the enzyme.

5. PIP_2 acts as an activator of mustard PLD as its incorporation into the substrate PC vesicles resulted in a great rise in the PLD activity, even in the presence of only micromolar levels of Ca^{2+} ions.

6. The mustard PLD has acidic pH optimum (pH 5.5-6.0) both in the mixed micellar as well as in aqueous-organic two phase system. It is moderately stable at a relatively broad pH range (pH 5.0-9.0) with the optimal stability at pH 8.5. The activation energy of the enzyme as calculated in the temperature range of 25-45°C is 8.8 kcal mol^{-1}. Similar to other plant PLDs, mustard PLD also possesses moderate thermal stability.

7. The activity in mixed micellar assay system is greatly influenced by the detergents. At all molar ratios of PC to detergent studied, anionic detergents like SDS and sodium deoxycholate strongly stimulated PLD activity with SDS displaying the highest stimulation effect. The enzyme also showed activity with cationic detergent like CTAB and non ionic detergent like Triton X-100. However, no activity was exhibited with non-ionic detergents like Tween 20 and Brij 58, respectively.

8. The phosphate analogs like AlF_4^- and Na_3VO_4, which are found to inhibit PLD activity in both animal and plant sources, also displayed strong inhibition of mustard PLD activity in a dose dependent manner. The AlF_4^- is a more potent inhibitor of the enzyme (IC_{50} = 7.8 µM) than Na_3VO_4 (IC_{50} = 33µM).

9. Mustard PLD exhibited hyperbolic v/[S] curve in both mixed micellar as well as two-phase assay system, when the activity was plotted as a function of substrate concentration. The subsequent V_{max} / K_m values for mixed micellar assay system and two-phase assay system were 356 ± 15 µmol$min^{-1}mg^{-1}$ / 1.84 ± 0.17 mM and 16.9 ± 1.8 µmol min^{-1} mg^{-1} / 0.96 ± 0.25 mM, respectively. The enzyme displayed different activity levels in mixed micellar and two-phase system as observed by the great differences in the kinetic parameters of the two assay systems for the same substrate soya PC. This implies that the substrate presentation to a lipolytic enzyme is an important parameter for the evaluation of its kinetic behavior.

10. The chemical structure of the phospholipid substrate has a profound effect on the activity of mustard PLD. The enzyme has a high catalytic efficiency towards phospholipids having short fatty acid chain (C8-C10) as compared to long ones (C12-C20). Also, it favored PC molecules with unsaturated fatty acids over saturated ones and the preference

depend upon the fatty acid chain length. The presence of both fatty acyl chains and their position on glycerol backbone greatly affects the enzyme activity as shown by its negligible activity towards soya Lyso PC and 1,3 PC molecular species, respectively.

11. With respect to alcoholic head groups of phospholipids, the enzyme showed a similar trend of substrate specificity in both the mixed micellar and two-phase system. In both the systems PC is the most favored substrate followed by PE and PG, however the level of hydrolysis of each phospholipid is different in both the assay systems. Though, PS is not hydrolyzed by the enzyme, it can be synthesized from PC in the transphosphatidylation reaction. Furthermore, the enzyme is not able to catalyze the conversion of PC into PI in the presence of *myo*-inositol, however it can cleave PI, although at very low level.

12. The properties of PLD purified from mustard seeds dramatically differ from the behavior of the PLD activity in the crude extract described previously (Khatoon *et al.,* 2007). From the present results we conclude that the enzyme represents a typical α-type PLD with the characteristics specified above.

8. References

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Declaration

I, the undersigned, declare that this dissertation is solely my own work and no part of it has been submitted to other Universities or Higher Learning Institutions. In addition, all sources of materials used in this dissertation have been duly acknowledged.

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